A Homogenous Fluorescence Assay of micro RNA Maturation

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Abstract

Micro RNAs (miRNAs) are non-coding double-stranded RNAs ~22 nucleotides long that play a vital role in development and regulation in nearly all eukaryotes. Through non-perfect hybridization to complementary sequences in the 3′-untranslated regions of mRNA protein translation is inhibited, leading to downregulation of the respective protein(s). Many diseases have been found to be influenced, if not caused (oncogenetic miRNAs) by aberrant expression of miRNAs. Thus, a manipulation of miRNA formation may have therapeutic potential.

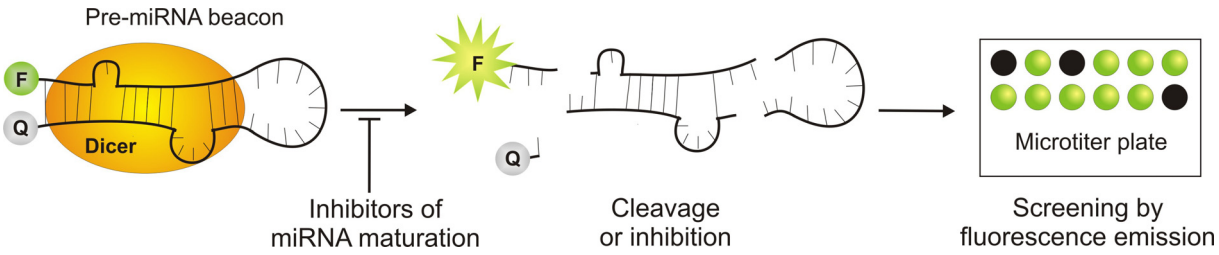
The miRNAs are cleaved from longer hairpin precursor RNA (pre-miRNA) in the cytoplasm by the enzyme Dicer. It might be possible to inhibit this enzymatic cleavage through specific pre-miRNA binding molecules, for example. Many compounds are known that bind RNA with high affinity such as aminoglycosides, proteins, peptides, and even other RNAs. Therefore, the search for selective binders of pre-miRNA and thus inhibitors of miRNA maturation begins with the synthesis and testing of large libraries of substances, best done using high throughput screening (HTS).

This work describes the first homogenous assay of miRNA maturation. The assay is based on a fluorescent probe in the form of a pre-miRNA containing a 5′-fluorophore (FAM, Cy3, or TMR) and a 3′-quencher (DABCYL). This pre-miRNA ‘beacon’ in its native hairpin formation brings the fluorophore and quencher moieties into close proximity, resulting in fluorescence quenching. Dicer enzyme is able to efficiently cleave this structure leading to dissociation of fluorophore and quencher and thus a concentration- and time-dependent fluorescence increase. In the presence of an RNA ligand that blocks Dicer from cleaving, a lower or no fluorescence increase is observed. The assay has been optimized for screening in 384-well microtiter plates using a plate reader so that HTS should be possible.

The first compounds were tested for their inhibition of pre-miRNA maturation by Dicer. Using a duplex assay with two different pre-miRNA probes each containing a different fluorogenic group (FAM or TMR) some specific inhibition could be shown. Additionally, the assay was performed in HEK 293 cells using fluorescence microscopy to detect the fluorescence increase. This would allow a cell-based screening of potential inhibitors.

In contrast to the first-generation pre-miRNA probes, which were made *via* semi-automated chemical RNA synthesis together with enzymatic ligation, an alternative approach using *in vitro* transcription followed by enzymatic ligation was established. Using various fluorophores and/or quenchers it should be possible to make a variety of beacons quickly and easily in large

amounts needed for HTS. Further development of the assay as well as synthesis and testing of compound libraries may lead to the discovery of highly selective inhibitors of pre-miRNA maturation and thus disease therapeutics.



Keywords:

miRNA, fluorescence assay, RNA binders, miRNA maturation inhibition

Zusammenfassung

Micro RNA (RNA = Ribonukleinsäuren) sind nicht-kodierende doppelsträngige RNA ~22 Nukleotiden lang, die eine wichtige Rolle in der Entwicklung und Regulation in beinahe allen Eukaryoten spielen. Durch unvollständige Hybridisierung mit komplementären Sequenzen im 3′-nicht-translatierten Bereich der mRNA wird die Proteintranslation inhibiert, was zu einer Herunterregulation des entsprechenden Proteins führt. Viele Krankheiten sind bekannt, die durch veränderte miRNA Expressionsmuster entweder beeinflusst oder sogar verursacht (onkogenetische miRNA) werden. Demnach könnte eine Manipulation der miRNA-Bildung einen therapeutischen Ansatz darstellen.

Die miRNA werden im Zytoplasma von längeren haarnadelförmigen prekursor RNA Strukturen (pre-miRNA) durch das Enzym Dicer freigesetzt. Inhibition dieser enzymatischen Spaltung könnte zum Beispiel durch spezifische pre-miRNA-bindende Moleküle erfolgen. Es sind viele Verbindungen bekannt die mit hoher Affinität RNA binden, wie Aminoglykoside, Proteine, Peptide und sogar andere RNA. Daher beginnt die Suche nach selektiven pre-miRNA Bindern und Inhibitoren der miRNA-Reifung mit der Synthese und dem Testen von großen Bibliotheken verschiedener Verbindungen bestenfalls durch Hochdurchsatzscreening.

Die vorliegende Arbeit beschreibt den ersten homogenen Assay der miRNA-Reifung. Der Assay basiert auf einer Fluoreszenzsonde in Form einer pre-miRNA, die einen 5′-Fluorophor (FAM, Cy3, oder TMR) und einen 3′-Quencher (DABCYL) aufweist. Durch die unmittelbare Nachbarschaft von Fluorophor und Quencher in der nativen Haarnadelstruktur der pre-miRNA-Sonde erfolgt Fluoreszenzlöschung. Das Enzym Dicer ist in der Lage diese Struktur effizient zu spalten, was wiederrum zur Dissoziation von Fluorophor und Quencher und somit zu einem konzentrations- und zeitabhängigen Fluoreszenzanstieg führt. Der Assay wurde in 384-Well Mikrotiterplatten optimiert, sodass ein Hochdurchsatzscreening möglich sein sollte.

Die ersten Verbindungen wurden mit dem Assay auf deren Inhibition der miRNA-Reifung bereits getestet. Unter Verwendung eines Duplexassays, wobei zwei unterschiedliche pre-miRNA Sonden mit verschiedenen Fluorophoren (FAM oder TMR) eingesetzt wurden, konnte etwas Spezifizität der Inhibition gezeigt werden. Außerdem wurde der Assay in HEK 293 Zellen durchgeführt, wobei der Fluoreszenzanstieg mit Fluoreszenzmikroskopie detektiert wurde. Somit ist ein Zell-basiertes Screening von potenziellen Inhibitoren möglich.

Im Gegensatz zur ersten Generation von Sonden, die über semi-automatisierte chemische RNA-Synthese zusammen mit enzymatischer Ligation hergestellet wurden, konnte eine

alternative Syntheseroute mit *in vitro* Transkription und anschließender enzymatischen Ligation entwickelt werden. Die Diversität der benötigten Sonden für ein Hochdurchsatzscreening kann durch den Einsatz unterschiedlicher Fluorophore und/oder Quencher bei der Sondenherstellung erreicht werden. Eine weitere Entwicklung des Assays, zusammen mit der Untersuchung von Substanzbibliotheken, sollte zu hoch selectiven Inhibitoren der pre-miRNA-Reifung und dadurch zu therapeutischen Ansätzen führen.

Schlagwörter:

miRNA, Fluoreszenzassay, RNA Binder, miRNA Reifung Inhibition

Table of Contents

Abbreviations

RNA and DNA are listed in the common format from 5′-terminus to 3′-terminus. Nucleobases are abbreviated using the common one-letter codes for RNA (G, A, C, U) or DNA (G, A, C, T).

ACN	Acetonitrile
ar	Aromatic
BMT	Benzylmercaptotetrazole
bp	Base pair(s)
calc.	Calculated
cpg	Controlled pore glass
Cy3	Cyanine 3
DABCYL	4-(Dimethylaminoazo)benzene-4-carboxylic acid
DCI	4,5-Dicyanoimidazole
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
D-MEM	Dulbecco's modified essential medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenyl
ds	Double-stranded
DTT	Dithiothreitol
EDTA	Ethylenediamine-N, N, N’, N’-tetraacetic acid
em	Emission
EtOH	Ethanol
ex	Excitation

FAM	Fluorescein
FC	Flash chromatography
FCS	Fetal calf serum
HIV	Human immunodeficiency virus
HTS	High throughput screening
IPA	Isopropyl alcohol
ITMS	Ion trap mass spectrometry
LNA	Locked-nucleic acid
LR	Low resolution
MA	Methylamine solution
MALDI-TOF	Matrix assisted laser desorption ionization – time of flight (spectrometry)
MeOH	Methanol
mer	Oligomer
min.	Minute
miRNA	Micro RNA
mRNA	Messenger RNA
N.A.	Numerical aperture
NaOAc	Sodium acetate
NH_4OAc	Ammonium acetate
NMR	Nuclear magnetic resonance (spectroscopy)
nt	Nucleotide(s)
PAGE	Polyacrylamide gel electrophoresis
PCI	Phenol /chloroform / isoamyl alcohol
PP	Polypropylene

Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PS	Polystyrene
Rev	Regulatory protein in HIV
R_f	Retention factor
rhDicer	Recombinant human Dicer
RNA	Ribonucleic acid
RNAi	RNA interference
RRE	Rev response element
rt	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SPR	Surface plasmon resonance (‘Biacore’)
ss	Single-stranded
SYBR Green / Gold	Cyanine-based nucleic acid stains
TAR	Transactivating response region
TBA	*tert*-Butylamine
TBAF	Tetrabutylammonium fluoride
TBDMS	*tert*-Butyldimethylsilyl-
TBE	Tris / borate / EDTA
TCA	Trichloroacetic acid

TEAA	Triethylammonium acetate
TEAB	Triethylammonium bicarbonate
TLC	Thin layer chromatography
T_m	'Melting' temperature at which 50% of ds nucleic acid is dissociated.
TMR	Carboxytetramethylrhodamine
TOM-	2'-*O*-Triisopropylsilyloxymethyl-
TPP	Thiamine pyrophosphate
t_R	Retention time
tRNA	Transfer RNA
U	Unit (activity of enzyme preparation)

1 Introduction

1.1 miRNA, siRNA and RNA Interference

The past fifteen years have seen a vast increase in interest in non-coding RNA (ncRNA). RNA was once believed to be more or less restricted to the transfer of the genetic information in the form of messenger RNA (mRNA). Several observations over this time, however, have greatly increased the understanding of the diverse roles of RNA in the cell.

It was in 1993 that the Ambros group discovered that a short ncRNA was responsible for regulation of a gene in the nematode *C. elegans*.[1, 2] Further work came to the conclusion that this RNA was actually only one member of a new class of RNAs [3, 4] that were then termed micro RNAs (miRNAs).[5, 6, 7] These RNAs ~22 bases long were found to bind complementarily to the mRNA, thus blocking protein translation. Micro RNAs are now known to be present in plants, animals, viruses and even algae.[8, 9, 10]

At about the same time work by Fire and Mello led to the discovery of what would become known as RNA interference (RNAi). Fire *el al* discovered that introduction of specific antisense RNA into *C. elegans* blocked mRNA translation to protein, even over multiple generations.[11] Work by Craig Mello and Andrew Fire then showed that long dsRNA injected into *C. elegans* can also cause suppression of gene expression, but at far lower concentrations.[12] RNAi had long before been observed in plants as a protective mechanism against viral infection[13] and is thought to play a similar role in animals (see also [14] and references therein). Eventually, it could be shown that RNAi is mediated by dsRNA ~22 base pairs (bp) long. Either long dsRNA or synthetic duplexes of 21-22 bp could mediate the silencing.[15]

Today RNAi describes the suppression of gene expression by both endogenous miRNAs as well as exogenous siRNAs. In plants and lower animals there are unique pathways, however, depending on the source of the short RNA.[16] The miRNAs generally bind with non-perfect complementarity to a given mRNA target sequence, causing translational inhibition. The siRNAs generally bind with perfect or near perfect complementarity to the respective sequence, which leads to cleavage and then degradation of the mRNA.

Within only a few years after its first observation, the use of RNAi in cultured cells was established as a very useful tool for determining the function of yet unknown genes identified by the human genome project without the need of the laborious knockout mouse technology. The importance that RNAi has attained in such a short period of time is highlighted by the

awarding of the Nobel Prize in Chemistry in 2006 to both Andrew Fire and Craig Mello for their work in this field.[17]

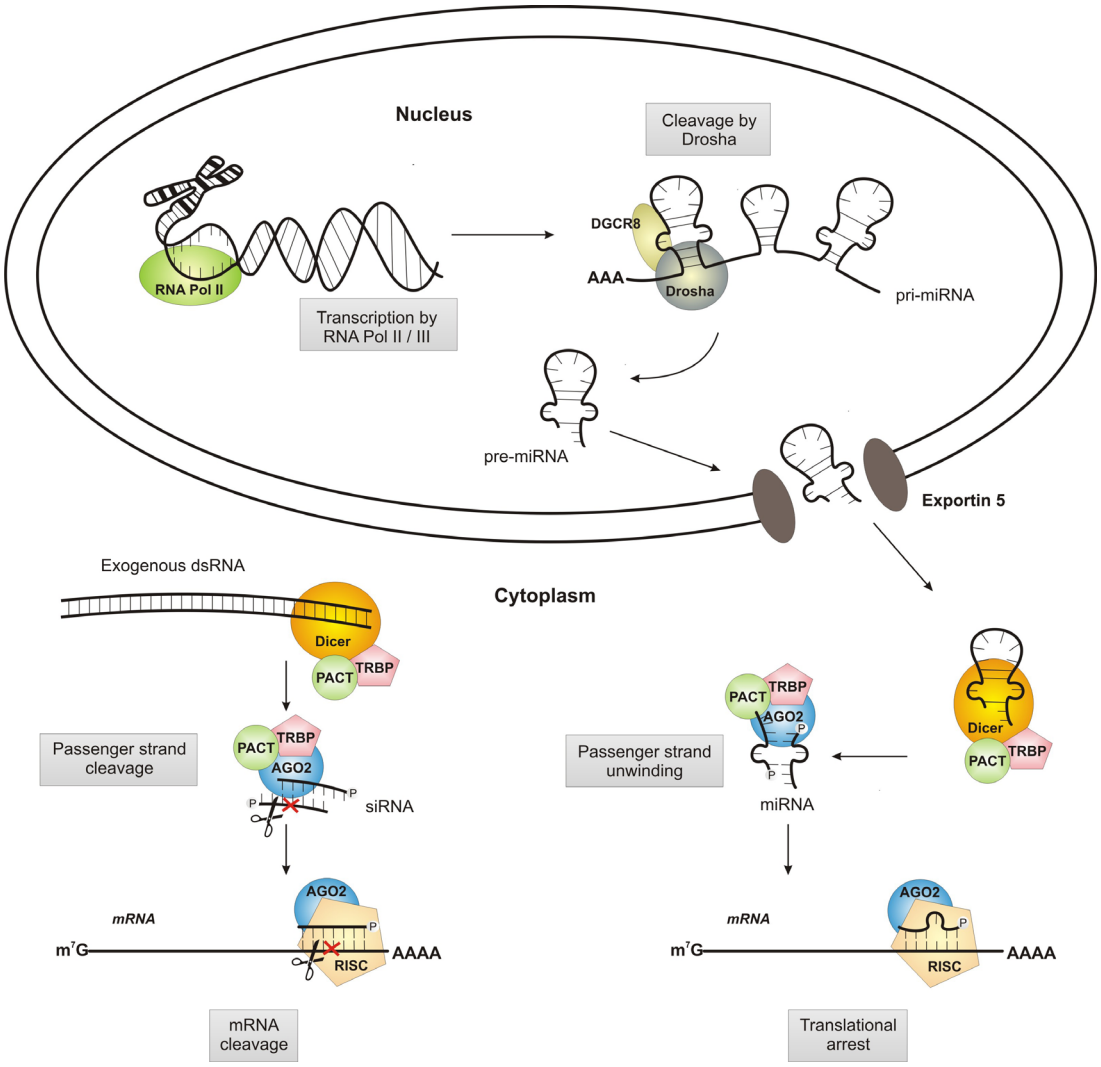


Figure 1: RNAi pathways for miRNA and siRNA in humans. RNA polymerase II/III transcribes long primary miRNA (pri-miRNA), which are cleaved by Drosha assisted by DGCR8. The resulting precursor miRNA (pre-miRNA) is exported from the nucleus by exportin 5 (right). The enzyme Dicer binds the pre-miRNA together with PACT and TRBP and cleaves the mature miRNA from the hairpin structure. The passenger strand is unwound and released. The guide strand together with AGO2 and various other proteins form an RNA-induced silencing complex (RISC) that binds with imperfect complementarity to the mRNA sequence resulting in translational arrest. In the siRNA pathway exogenous dsRNA is processed by Dicer as described (left), producing the siRNA. AGO2 joins the complex and cleaves the passenger strand and the guide strand is loaded into the RISC. AGO2 then becomes part of the RISC and once again cleaves the complementary gene sequence in the mRNA, resulting in mRNA degradation and thus loss of gene expression.

A growing body of data suggests that the miRNA and siRNA pathways in humans are closely connected. The miRNA pathway begins in the nucleus where usually RNA polymerase II, [18] but also Pol III [19] produce long polycistronic primary miRNA (pri-miRNA) transcripts (Figure 1). In humans the RNAse III class enzyme Drosha in sync with the protein DGCR8 (DiGeorge syndrome critical region gene 8) cleaves RNA hairpins ~70 bp long from much longer primary stem-loop structures in the pri-miRNA.[20, 21] These precursor miRNA (pre-miRNA) hairpin RNAs are then transported to the cytoplasm by the protein exportin 5.[22]

In the cytoplasm the pre-miRNAs are cleaved by the enzyme Dicer ([23], see also [24] and references therein) to give the active miRNA duplex. Exogenous dsRNA is also cleaved by Dicer to form siRNAs. The miRNAs (or siRNAs) generally contain an antisense 'guide' strand, which is complementary to the target RNA as well as a sense 'passenger' strand, although in some cases both strands are known to be active. The strand with greater duplex stability at its 5′-terminus becomes the antisense 'guide' strand.[25, 26]

After cleavage by Dicer the miRNA duplex is transferred directly into a ribonucleoprotein complex called RISC (RNA-induced silencing complex). The human immunodeficiency virus transactivating response RNA-binding protein (TRBP) in tandem with the protein activator of protein kinase (PACT) seem essential in this mechanism.[27, 28, 29] Both proteins aid human Dicer in binding and cleaving dsRNA. [30] It appears that these proteins also enable transfer of the siRNA / miRNA to the RISC. It is possible that PACT takes on this role for miRNA whereas TRBP is the active protein in loading siRNA into the RISC.[29] A recent report showed that *in vitro* reconstitution of recombinant human Dicer with TRBP and AGO2 resulted in spontaneous formation of a complex capable of pre-miRNA cleavage, guide strand loading to Argonaute 2, and cleavage of a perfect complementary target RNA.[31] This shows not only loading of the proper antisense strand, but also activation of the Argonaute 2 protein. In the siRNA pathway, the Argonaut protein 2 (AGO 2) is responsible for cleavage of the target mRNA.[32]

In the miRNA pathway, non-perfect binding of the guide strand to the complementary sequence in the 3′-untranslated regions (UTR) of the mRNA results in translational inhibition by steric blockage rather than mRNA degradation. However, subsequent removal of the respective mRNA to processing bodies (P-bodies) takes place where degradation can occur.[33] The 'seed' region of the miRNA must contain perfect 5′-complementarity with its target sequence in the first 2-7 nt.[34] In some cases both strands in the miRNA can be

functional, which adds confusion to the exact mechanism of this interference pathway. Perhaps in such cases translational repression occurs with random insertion of one miRNA strand or the other into the RISC. As mentioned already siRNA bind with perfect complementarity to their target sequences, resulting in cleavage of the mRNA in the RISC by AGO2.

1.2 Dicer

Human Dicer is a multi-domain protein ~220 kDa long and a member of the RNAse III class of enzymes. It is made up of a DExH RNA helicase / ATPase domain, a domain of unknown function (DUF283), a PAZ (Piwi Argonaute Zwille) domain, two RNase III-like domains (RIIIa and RIIIb), as well as a dsRNA binding domain (dsRBD) (Figure 2).[23] The helicase domain is believed to aid in unwinding of the miRNA duplex, although Dicer-mediated cleavage itself seems to be independent of ATP.[35, 36, 37] The DUF283 might also play a dsRNA binding role.[38] Further, the two RNAse II-like domains are responsible for independent cleavage each of one side of the dsRNA.[39]

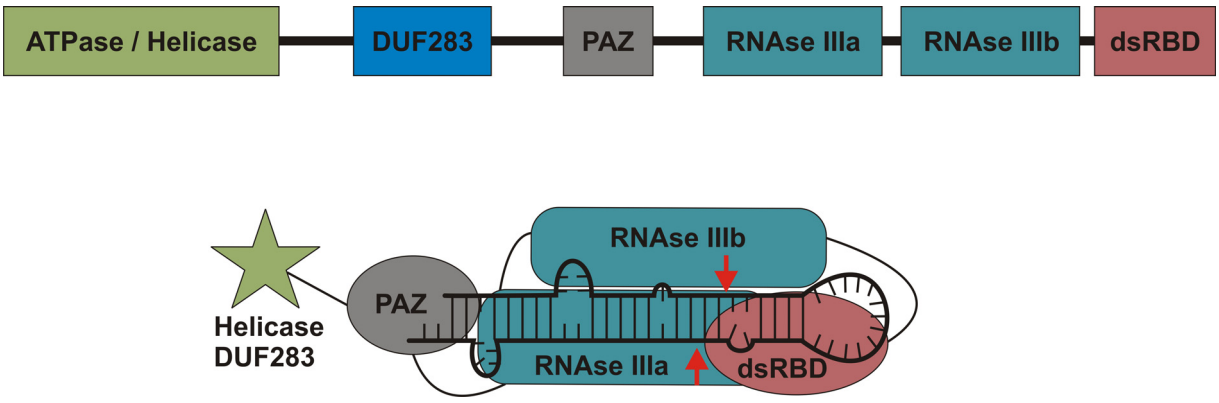


Figure 2: Dicer domains (top) together with its cleavage mechanism (bottom).[39]

The crystal structure of Dicer from *Giardia intenstinalis* provides some insight into the mechanism of Dicer cleavage including the role of the PAZ domain as a molecular ruler.[40, 41] Recently, a C-terminal fragment from human Dicer containing the RNAse IIIb domain was solved.[42] This revealed magnesium cations at the active center, which most likely act as catalysts in the cleavage mechanism in human Dicer. A structure of the RNAse IIIb domain together with the dsRBD from mouse Dicer [43] showed that a conserved lysine residue

stabilizes the cleavage transition state at the scissile phosphodiester bond. This is believed to take place *via* the S_N2 nucleophilic attack of a water molecule activated by Mg^{2+} coordinated by four carboxylate residues.[44]

In *C. elegans* and mammals there is only one Dicer enzyme known. The plant *A. thaliana*, for example, possesses four different Dicer paralogues, while *D. melanogaster* (fruit fly) has two unique Dicer enzymes, each responsible for processing dsRNA from different sources (dsRNA or pre-miRNA). It is possible that a yet unknown protein in *C. elegans* and mammals helps their single Dicer enzyme distinguish between different sources of RNA.[16]

Human Dicer cleaves not only the pre-miRNAs to give miRNA, but also longer exogenous dsRNA to produce siRNA. In either case Dicer cleaves at a typical distance of ~22 bases from the open end of the RNA (Figure 2). The pre-miRNAs contain 5′-phosphates and 3′-overhangs of 2 nt, which is characteristic of cleavage products from the RNAse III class of enzymes. The PAZ domain of Dicer recognizes the 3′-overhang while measuring like a molecular ruler a fixed length along the RNA at which to cut.[39, 41, 45] The active miRNA then also contains typical 5′-phosphates and 3′-overhangs of 2 nt.

Although various 3′-overhang lengths are recognized by Dicer, any overhang 2-5 nt results in cleavage lengths < 24 nt, with a reduction in cleavage efficiency observed with increasing overhang length. With structures containing 0-1 nt 3′-overhangs a primary cleavage of 25-26 mers occurs followed by a secondary cleavage of these structures to give 21-24 mer (as well as 2-5 nt) products.[46] A similar phenomenon was observed with *Giardia intestinales* when a 5′-overhang of 2 nt instead of a 3′-overhang is present. This results in a cleavage product ~4 nt longer (29 mer) than the normal 25 nt.[47]

1.3 miRNA Function and Disease

Hundreds of publications over the past several years suggest a key role of miRNAs in development and regulation in nearly all organisms. Alone in humans 400-500 miRNAs have been discovered with up to 1000 expected to be found.[48] Up to 30% of the human genome is thought to be regulated by miRNAs.[34] Detection of miRNAs has largely been done using Northern blotting as the ‘gold standard’, however many methods have been developed in recent years for high throughput screening including microarrays, reverse transcription PCR (RT-PCR) as well as bioinformatic approaches. The most modern techniques allow detection into the low femtomolar range.[49]

The first miRNAs to be widely studied, lin-4 (lineage-4) and let-7 (lethal-7), were found to play a decisive role in determining the timing of larval development in *C. elegans*.[1, 4] Bantam, another well studied example from *D. melanogaster*, together with mir-2, mir-6, and mir-14 regulate tissue growth by modulating apoptosis and cell proliferation.[50] The importance of miRNAs during developmental stages is becoming ever more detailed, particularly in humans where their exact functions were widely unknown.[51, 52] It is now believed that a single miRNA is responsible for regulating various cellular functions because of incomplete base pairing to different mRNAs.[52] In humans, mir-15 and mir-16 have been found to play a role in determining the embryonic axis, for example. Mir-124 is a possible contributor to neuronal differentiation.[50]

The role of miRNA has also been associated with disease etiology. For example, the BIC gene (B-cell integration cluster) encodes mir-155, which has been found to be overexpressed in various lymphomas and breast and lung carcinoma.[50, 53] Mir-155 is also known to be upregulated in the macrophage inflammatory response, perhaps linking it between inflammation and cancer.[54] The *ras* gene is regulated by the let-7 miRNA family, underexpression of which is accompanied by overexpression of RAS protein in lung tumors.[55] Constitutive signaling of the RAS-pathway leads to stimulated cell growth and repressed apoptosis.[56] Micro RNAs have also been recently associated with Parkinson's disease, [57, 58] Alzheimer's disease, [59] and hypertrophy of the heart.[60] In general, impaired miRNA processing is known to promote cellular transformation and tumorigenesis.[61, 62]

Many more miRNAs have been found to be deregulated in various disease states, particularly in cancer (for some recent reviews see [63, 64, 65, 66]). Mir-15 and mir-16 are deleted or downregulated in ~68% of all chronic lymphocytic leukemia cases.[67] The mir-17-92 cluster of miRNAs is upregulated in lymphomas [68] and lung cancer [69] and purported to be an oncogene. Other miRNAs also believed to act as oncogenes are mir-21 in brain [70] and breast [71, 72] cancers and the aforementioned mir-155.

The exact mechanisms of how miRNAs are involved in gene regulation and pathogenesis are still widely unknown. However, the ever growing list of miRNAs found to be involved in disease processes calls for the development miRNA-directed therapies.

1.4 Therapeutic Possibilities in RNAi Pathways

1.4.1 siRNA and Antisense miRNA 'Antagomirs'

Since the discovery of RNAi with long dsRNA [12] many groups around the world eagerly moved from using siRNAs to produce knockdown phenotypes in experimental models [73] towards using siRNAs as potential therapies, especially in cancer treatment. Very simply, the antisense strand of an siRNA duplex should bind perfectly to its target mRNA, resulting in downregulation of the respective protein(s) through destruction of the respective mRNA and may lead to an improvement in the condition of patient. But the use of siRNA as a therapeutic is not trivial owing to the difficulties in production and administration of the drug.[74]

The idea of disease treatment using antisense nucleic acids to block translation of mRNA has been around for many years. Pathologically overexpressed proteins can be easily reduced in cell culture using antisense molecules against the respective mRNA, thus blocking protein translation. Unfortunately, nucleic acids have poor pharmacokinetic properties. Because of this the only antisense drug that has made it to the market is Vitravene® (Fomivirsen sodium) from ISIS Pharmaceuticals for treating cytomegalovirus-induced retinitis. This drug only acts topically in the eyes and up to now there are no systemically-acting antisense molecules on the market.[75] However, the discovery of RNA interference in recent years has added impulse to the development of improved antisense drugs. Various antisense molecules against RNA are currently in human phase II and III trials.[76]

The synthesis, packaging, and particularly delivery of an siRNA drug is also difficult, with the intravenous route being the only viable option to date for systemic treatment.[77] Additionally, off-target effects as well as induction of the innate immune system complicate the therapeutic effectiveness of siRNA.[74] Although RNA can be synthesized in large amounts needed for such treatments, there are very high costs in terms of manufacturing and to the environment in terms of the amount of waste produced through solid-phase synthesis. Since RNA is degraded easily by RNAses (enzymes specific for RNA), which are found on every person's fingertips, packaging and storage of the final drug is critical to assure stability. Once administrated, the RNA is also susceptible to RNAse degradation in the body. And finally, the siRNA must be appropriately delivered, so as to assure uptake of these very polar molecules. Liposomal delivery methods are currently being developed among others.[78] Modifications of the RNA itself (phosphorthioate backbone, 2′-OMe protection, carriers, etc.) can also be made in order to increase uptake and stability of the drug.[74, 77]

Despite the various limitations caused by poor pharmacokinetic properties and off-target effects, many companies are currently developing siRNA treatments for a wide range of diseases.[79] Additionally, more viable introduction of the active siRNA can be achieved by a 'gene therapy' approach using retroviral transformation and transfection of a plasmid containing the desired sequence directly into human cells. In the form of synthetic hairpin RNA (shRNA), processing by Dicer results in much greater therapeutic efficacy.[74]

Alternatively, regulation of miRNAs themselves can also be envisioned.[80] A good example of blocking miRNA function was shown by Krützfeld *et al.*[81, 82] They synthesized cholesterol-labeled 21-23 mer antisense RNA complementary to one of the strands of miRNAs and introduced these so-called 'antagomirs' into mice (Figure 3). This resulted in reduction of the corresponding miRNA levels in various tissues, in contrast to unmodified RNA. One antagomir against mir-122 resulted in elevated levels of the mRNA that is targeted by the endogenous mir-122. In these animals a 40% reduction in plasma cholesterol levels was also observed, proving the link between mir-122 and the cholesterol biosynthetic pathway. The reduction in protein levels involved in cholesterol metabolism was only examined for a few enzymes, however, and the prolonged effect of reduced levels of mir-122 is currently unclear.[83]

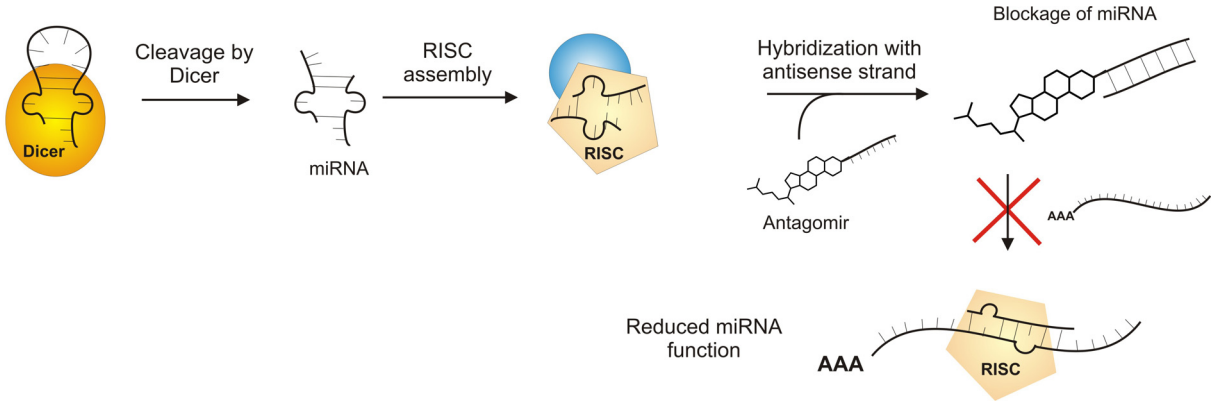


Figure 3: Binding of miRNA by complementary antagomirs leads to inhibition of miRNA function.

1.4.2 Small Molecules, Peptides, and Aptamers as RNA Binders

Other approaches towards regulating miRNAs would be through synthetic 'small molecules' (typically < 500 Da for orally active compounds), [84] peptides or even aptamers. Smaller organic molecules have general advantages over biologics including greater stability, easier

manufacturing and packaging (less expense), and usually easier delivery and administration (including orally).

RNA has been a target of medical chemistry for a very long time. In the search for treatments of infectious diseases systematic screening revealed the aminoglycosides as potent antibiotics and binders of bacterial ribosomal RNA. The binding of ribosomal RNA causes misreading of the genetic information or inhibition of protein translation.[85, 86, 87] Although aminoglycosides can have fairly severe side effects, they remain the chosen therapy for serious bacterial infections such as tuberculosis and bacterial meningitis.[88]

In the last five to ten years viral RNA has also become a target for drug development. The HIV-1 viral genome has been of particular interest in the search for binders of its trans-activating response (TAR) RNA [89, 90] and the Rev-RRE (Rev response element) region.[91, 92] Much research has been also done into targeting the internal ribosomal entry site (IRES) of the hepatitis C virus (HCV).[93] Important to note is that the targeted RNA regions are made up of hairpins, often with multiple unpaired regions, which are potential binding epitopes for RNA ligands.

Other non-aminoglycosidic antibiotics are also known to bind ribosomal RNA including tetracycline und spectinomycin. A particularly interesting example of an RNA ligand is tylophorine B (Figure 4). Although intercalation in DNA or RNA could be expected based on the phenanthrene ring system it has been recently shown that tylophorine B is a selective bulge binder in double-stranded DNA [94] and RNA.[95]

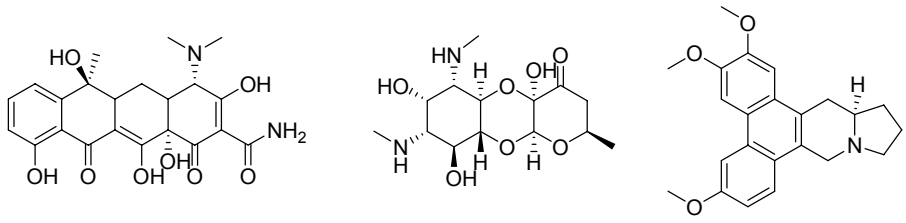


Figure 4: Antibiotics tetracycline (left), spectinomycin (middle), and the DNA/RNA bulge binder tylophorine B (right).

Aminoglycosides have been very often used as scaffolds to synthesize novel compounds with greater selectivity and potency. Modifications include the systematic substitution of amino and hydroxyl groups, [96, 97, 98] substitution of amino groups with the more basic guanidino group, [99] formation of aminoglycoside acridine conjugates capable of intercalation [100] as

well as the dimerization of aminoglycosides (Figure 5 and Figure 6). [101, 102, 103, 104, 105, 106]

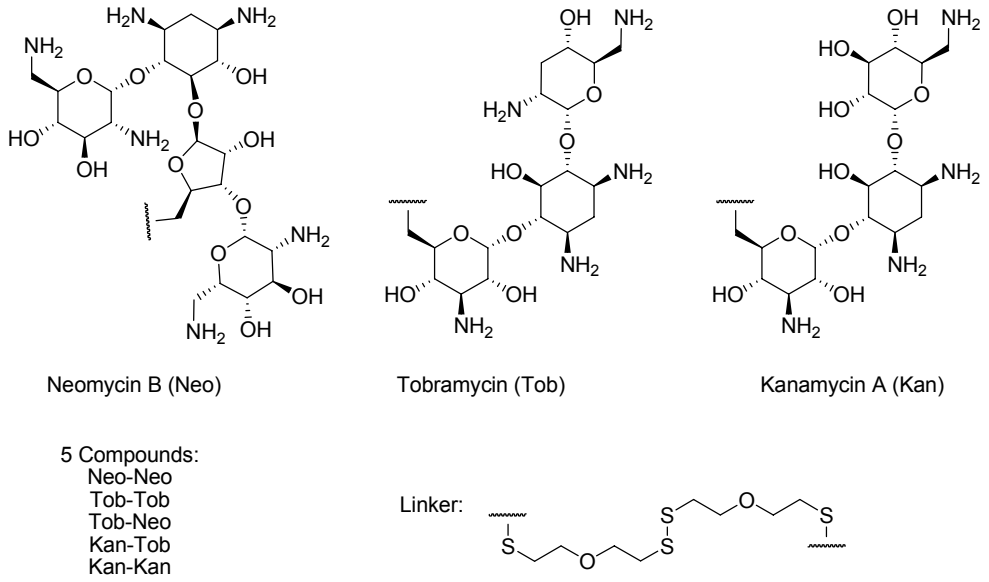


Figure 5: Dimerized aminoglycosides synthesized by Tor and coworkers.

Multivalency is a very important concept in ligand binding. The ability to achieve much higher binding affinity (usually greater than simply the sum of effects of the individual ligands) through binding of multiple epitopes has far reaching consequences.[107] Foreseeably, stronger binding would translate to greater inhibitory effect and enhanced selectivity resulting in far lower dosing of a drug being needed and thus, possibly, lower side effects.

Considerable progress has been made with dimerized aminoglycosides, however the synthetic routes to such molecules is very laborious. The development of large libraries for screening potential ligands of new target RNA structures has also not been possible. Diverse reports describe therefore the synthesis of modified carbohydrates and aminosugars, which are easier to make synthetically and are more suited to developing large compound libraries.[108]

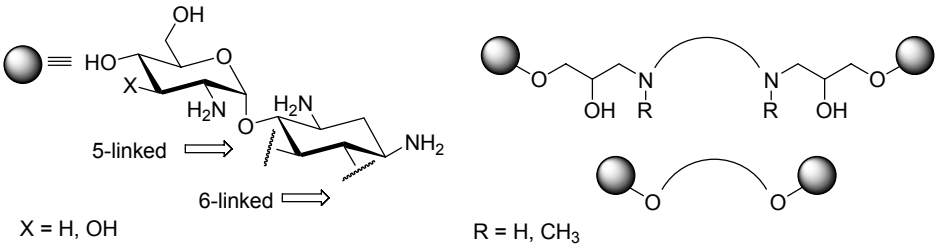


Figure 6: A total of twelve neamine dimers were synthesized by the Wong group.

A good example showing the development of RNA binding molecules was initiated by the Hergenrother group. They dimerized the known 2-deoxystreptamine (2-DOS) building block with a variety of structurally diverse linkers to obtain a small library of RNA binders (13 compounds).[109] The synthetic strategy was extended using the Sharpless-Meldal variant of the copper-catalyzed 1,3-dipolar cycloaddition (Huisgen reaction) of organic azides on terminal alkynes. The ease of use of this reaction (chemoselectivity and high yields) led to the term 'click chemistry'.[110] In combination with solid-phase-based purification Hergenrother *et al* were able to synthesize 105 dimeric 2-DOS-conjugates, three of which showed size-specific rather than sequence-specific affinity for different synthetic RNA hairpins.[111] Although RNAse footprinting assays revealed binding to the hairpin terminal loops the exact type of binding between these ligands and the RNA remains unclear, particularly why the use of such similar linkers resulted in so very different binding properties.[112]

Peptides are also good candidates as RNA ligands.[113, 114] The synthesis of peptide RNA-binders is advantageous because of well-established solid-phase and protecting-group techniques. Large libraries of compounds can be synthesized fully automatically with relative ease. For example, cyclic peptide mimics of the Arg-rich motif in the HIV-1 Tat protein were found to be potent RRE binders.[115] Acridine modified helical peptides were found to have picomolar binding affinities towards both RRE and TAR RNA.[116]. Recently, binding of a macrocyclic peptide was even shown for pre-mir-23b at 2.3 μM with selectivity for other artificial RNA hairpins.[117]

The difficulty in discovering new peptide-based RNA binders lies in appropriate assays for their detection. In the search for inhibitors of Tat-TAR and Rev-RRE protein-RNA interactions fluorescence-based binding assays (fluorescence quenching) and fluorescence anisotropy assays have been used.[118] Another promising method is the split-mix synthesis of peptide libraries (one bead, one compound) where RNA binding of the peptide takes place directly on solid phase and is detected *via* binding of dye-labeled RNA.[119] The latter method requires sequencing of the polymer-bound peptides, which can be particularly difficult when using modified amino acids. However, since such 'split-mix' peptide libraries have nearly no size limit it is logical to use only those amino acids that would be expected to possess good RNA-binding properties. With this goal in mind non-natural amino acids containing heteroaromatic side chains were synthesized.[117, 120, 121, 122] Dissociation constants between 10^{-8} and 10^{-9} M can be achieved. The selectivity over other RNAs (e.g. tRNA), however, often differs by only one order of magnitude.

Most exciting is the ability of RNA aptamers to bind RNA.[123] Through the use of systematic evolution of ligands by exponential enrichment (SELEX) high affinity RNA or DNA binders of a desired target can be made. This is achieved, as the name suggests, though repeated selection cycles for the desired target starting from a large random pool of synthetic DNA (10^{15}).[124, 125, 126] The DNA can then be transcribed into RNA when desired. Famulok and coworkers have been able to identify RNA aptamers that bind the TPP riboswitch.[127] The Toulmé group has intensely studied 'kissing complexes' whereby terminal loops of RNA hairpins interact with each other. They have developed RNA aptamers that bind the HIV-1 TAR RNA with high affinity. Recently, a locked-nucleic acid (LNA) aptamer was developed having picomolar binding affinities to TAR RNA.[128] Linkage of the 2′-hydroxyl *via* a methylene bridge to the 4′-position locks the RNA in the preferred 3′-*endo* conformation resulting in substantially increased resistance to degradation as well as increased binding affinities.

Another highly intriguing method to stabilize RNA aptamers is through the use of *Spiegelmers* (*Spiegel* = mirror).[129, 130] A known peptide target is synthesized using the unnatural D-amino acids. SELEX is then performed against this target using natural RNA (D-isomers). Owing to chirality in nature the L-peptide target then binds with the same affinity to the unnatural L-RNA. Spigelmers are not only high affinity binders of their targets, but virtually completely resistant to nucleases. Important is that aptamers, including spiegelmers, show virtually no adverse immune response in rabbits.[131]

1.5 Inhibition of miRNA Maturation as a Therapeutic Concept

Although RNA in the cell is usually single-stranded it can fold back on itself producing typical secondary structural elements to which ligands can bind (A-helix, hairpins, bulges, loops). Such structures are also seen with pre-miRNAs (Figure 7). Small molecules can bind the various bulges and loops. Accordingly, linking of such molecules could increase their binding affinities. However, even with such diverse structures to which molecules might bind, the design of selective RNA ligands is still very difficult because secondary and tertiary RNA structures cannot be properly predicted.[132]

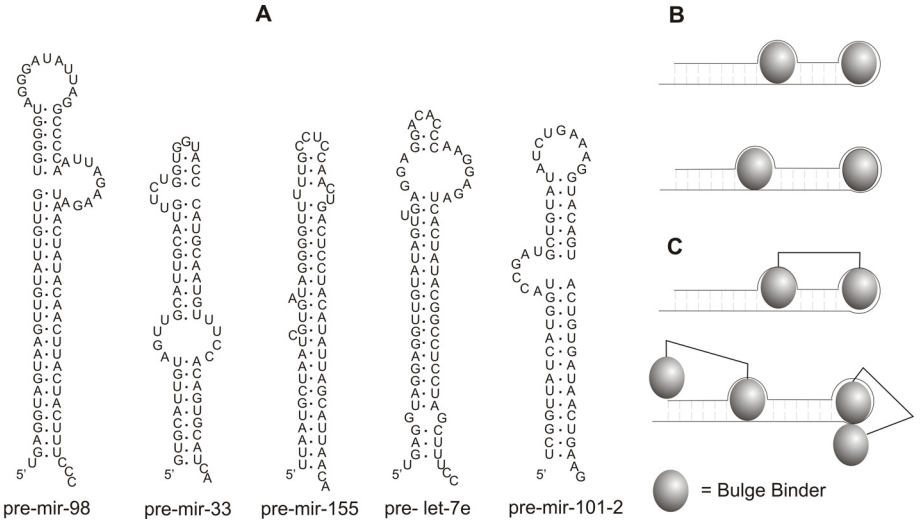


Figure 7: A) Calculated secondary structures of various human pre-miRNAs predicted using RNAstructure 4.5 software.[133] B) Binding of structural elements by small molecule bulge binders. C) Increased inhibition may be achieved by connecting ligands *via* a linker.

Recently, An *et al* were able to inhibit the Dicer-mediated cleavage of an shRNA containing an aptamer for theophylline, which was fused to the loop region of the shRNA.[134] The shRNA stem coded for an siRNA against EGFP. Addition of theophylline resulted in a dose-dependent inhibition of EGFP silencing. Similar constructs with the expected Dicer cleavage site shifted by 1 or 2 nt outside of the theophylline aptamer resulted in less or no response to theophylline, respectively. This shows that Dicer activity is blocked by a small molecule, which binds the pre-miRNA at the Dicer cleavage site. This shows that simply monitoring the binding of small molecules to a pre-miRNA would not provide information about the inhibition of miRNA maturation.

Micro RNAs are known to play a vital role in development and regulation in many species and their deregulation has been connected with various diseases including cancer. Thus, miRNAs or their precursor molecules the pre-miRNAs are potential targets for treatment in diseases where their misexpression is known to contribute to the disease state. Inhibition of miRNA maturation using specific RNA binders might be desirable, for example, when an overexpressed miRNA contributes to a given disease process. To study RNA-ligand interactions, however, an appropriate assay is required.

2 Objectives

In order to study potential inhibitors of miRNA maturation a homogenous fluorescence assay was envisioned based on the idea of 'molecular beacons' used in nucleic acid detection.[135, 136] The 'beacon' in this case would be a pre-miRNA labeled with a fluorophore at the 5′-terminus and a quencher at the 3′-terminus, their spatial proximity to one another resulting in a quenched state. In the presence of Dicer the labeled miRNA maturation probe would be cleaved, releasing the fluorophore and resulting in a fluorescence increase. However, when a binder of pre-miRNA or Dicer is also present the probe would not be cleaved and no fluorescence increase would be seen (Figure 8). This is analogous to common assay designs for protease inhibitors.[137]

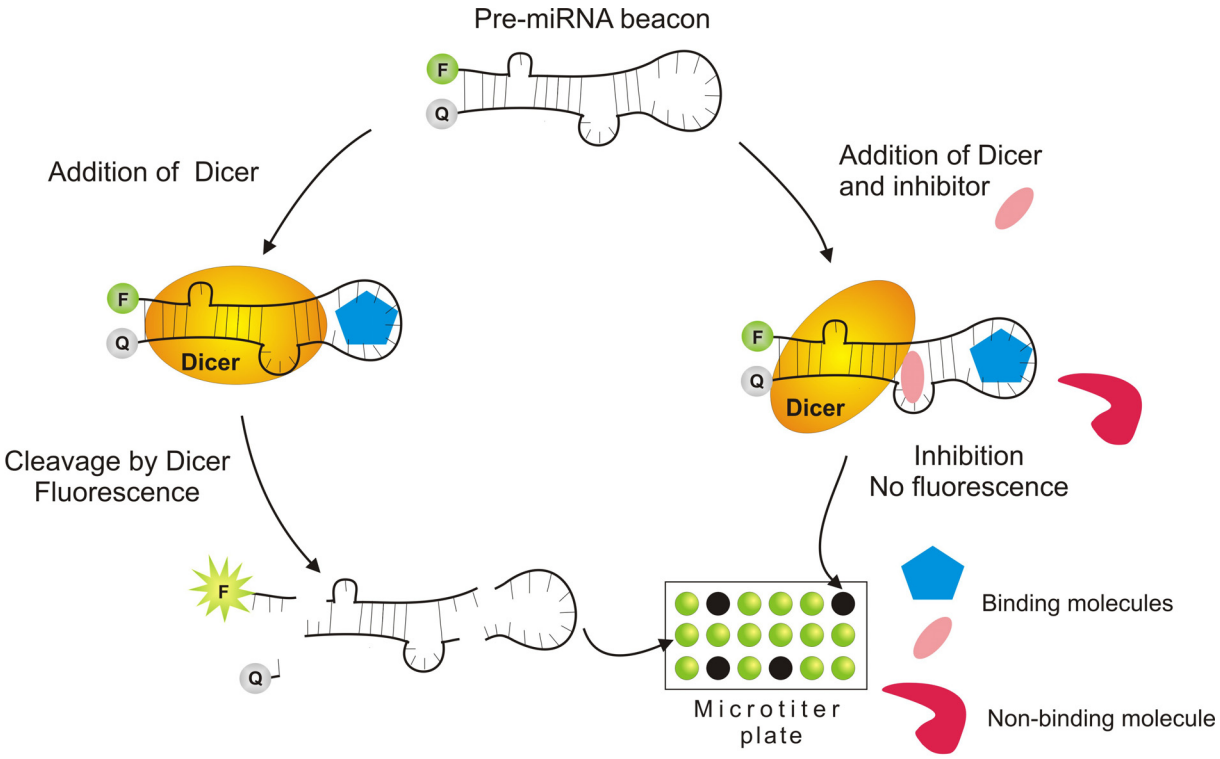


Figure 8: Micro RNA maturation assay. Probe cleaved by Dicer (left) results in fluorescence increase. In the presence of the proper ligand, Dicer cleavage is blocked (right). Some molecules may bind (blue polygon) that do not inhibit Dicer cleavage.

Such a homogenous assay would ideally allow simple and fast detection of pre-miRNA binders, which inhibit cleavage by Dicer. The advantage of such an assay is that it would provide a direct method of measuring the inhibitory effect of a given test substance on the Dicer-mediated cleavage of a pre-miRNA in real-time. This would allow a preselection of

specific inhibitors before cell-based assays are used. It could be applied in a high throughput screening (HTS) format. Easy fluorescence readout would make it practical and sensitive. A large number of fluorophores are available to select from and synthesis of such a miRNA maturation probe could be achieved through chemical synthesis or *in vitro* transcription.

The objectives of this work were to first establish a 'proof of principle' of the assay. Transfer of the assay to a HTS format was then desired to allow testing of libraries of potential pre-miRNA binders.

3 Results and Discussion

3.1 Synthesis of Labeled miRNA Maturation Probe

3.1.1 Chemical Synthesis of RNA Strands

The various strands needed in order to make the final beacons were synthesized using semi-automated RNA synthesis on cpg or polystyrene supports. Unmodified RNA was first synthesized automatically on a nucleic acid synthesizer and 5′-modifications were then manually coupled to ensure greatest yields. For 3′-DABCYL strands the DABCYL modification was first coupled by hand to the polystyrene support followed by automated RNA synthesis (Figure 9). The TOM-protecting group chemistry (TOM ≡ 2'-*O*-triisopropylsilyloxymethyl) was chosen as it had been shown to yield purer RNA in higher yields as opposed to standard 2′-TBDMS chemistry.[138, 139, 140, 141] According to the literature benzylmercaptotetrazole (BMT) was used as the phosphoramidite activator.

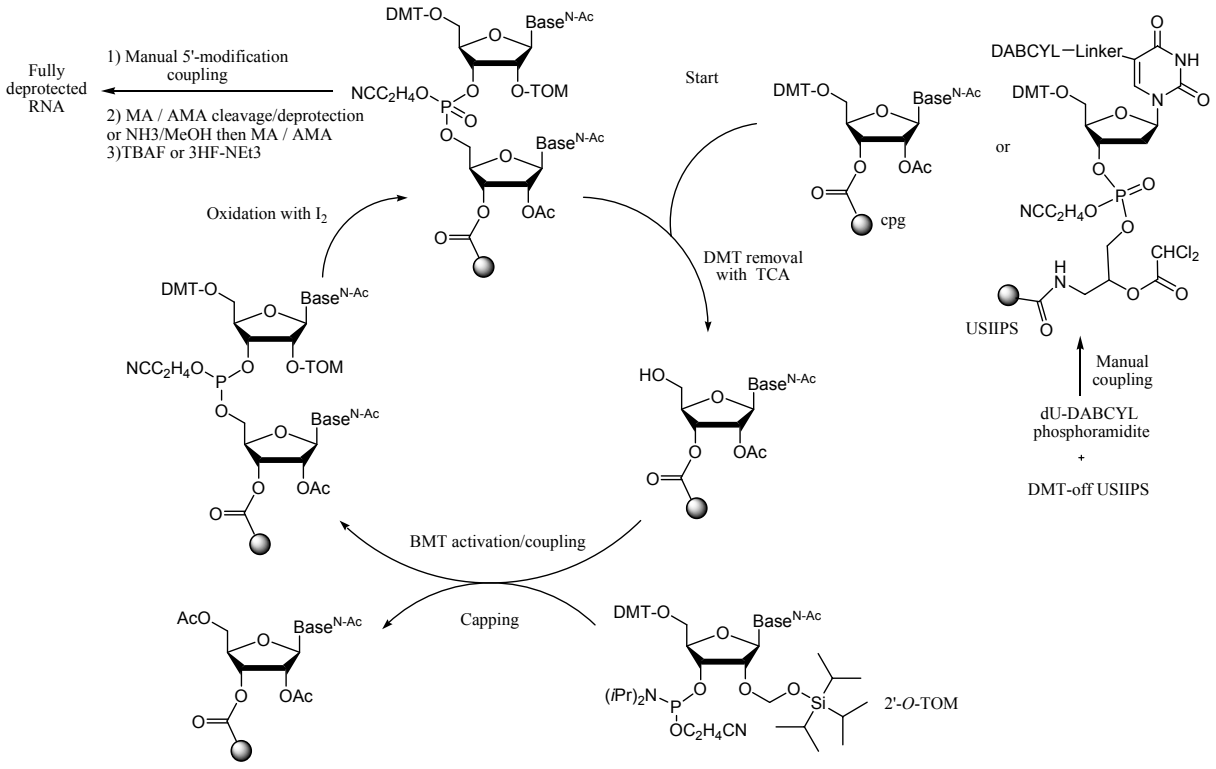


Figure 9: Semi-automated RNA synthetic route. Bases used are N^6-acetyladenine, N^2-acetylguanine, N^4-acetylcytosine, and uracil. Adapted from [140].

The utilization here of the FAM and DABCYL groups proved compatible with the 2′-TOM-protection group chemistry (Figure 10). It was found that either methylamine solution (MA, 35 °C for 6 hrs) or ammonium hydroxide / methylamine solution (AMA, for 2 hrs at rt) could be used for cpg and base / phosphate protecting-group cleavage equally well with similar yields. Even the Cy3 modification was found to be relatively stable in the AMA solution for up to 2 hours. Longer incubation times did result in some degradation.

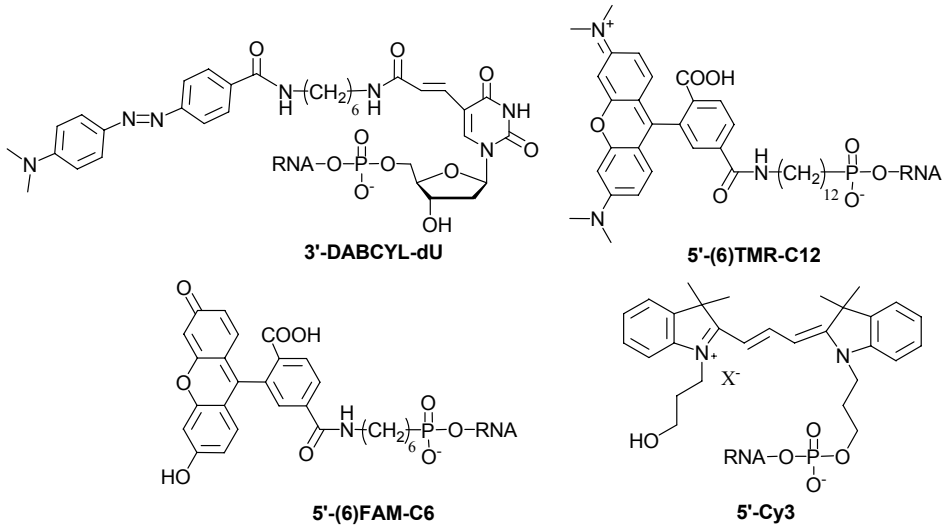


Figure 10: Dyes used in synthetic beacons.

The use of the universal polystyrene support II (USIIPS) for the DABCYL strands proved very convenient and high yielding, allowing synthesis of a 3′-DABCYL-modified RNA containing a 3′-OH group. Cleavage from support using dry ammonia / MeOH followed by additional base deprotection in either MA or AMA was very effective as seen in HPLC traces and yields.

The 2′-deprotection step using TBAF was at least compatible with the FAM, DABCYL and Cy3 groups. Additionally, the later use of triethylamine trihydrofluoride for 2′-desilylation was also fully compatible with the FAM, DABCYL, and TMR groups. This method was preferred over the literature recommended TBAF deprotection as the final deprotected product could be more easily alcohol precipitated without an additional desalting step as recommended with TBAF cleavage of the TOM protecting group.[139] A desalting step after TBAF deprotection was also avoided by using alcohol precipitation to isolate the final deprotected products, however, more salt must be removed by rinsing with 70% EtOH. In either case, more depurination was observed in MALDI-TOF analysis of the fully deprotected

3′-DABCYL strands than the 5′-fluorophore-containing strands. In total 9 RNA strands were synthesized for use in the formation of various beacons (Table 1).

Table 1: Synthetic RNA strands used for beacon ligations.

Strands for FAM pre-mir-142 beacons	
5′-FAM 32 mer (1)	5′-FAM-CAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′
5′-FAM 33 mer (2)	5′-FAM-CCAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′
3′-DABCYL 27 mer (3)	5′-PO_3-AGGGUGUAGUGUUUCCUACUUUAUGGdU-DABCYL-3′
Strands for Cy3 and TMR pre-mir-19b-2 beacons	
5′-Cy3 31 mer (4)	5′-Cy3-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′
5′-Cy3 32 mer (5)	5’-Cy3-CAGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′
5′-TMR 31 mer (6)	5′-TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′
3′-DABCYL, 35 mer (7)	5′-PO_3-UGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′
Strands for FAM pre-bantam beacon	
5′-FAM 30 mer (8)	5′-FAM-CCGGUUUUCGAUUUGGUUUGACUGUUUUUC-OH-3′
5′-DABCYL 30 mer (9)	5′-PO_3-AUACAAGUGAGAUCAUUUUGAAAGCUGAUdU-DABCYL-3′

Unfortunately, synthesis of 5′-TMR-modified RNA was completely incompatible with 2′-TOM-protecting group chemistry and deprotection scheme. Rhodamine dyes are sensitive to the typically harsh deprotection strategies used in DNA / RNA synthesis. It was seen here that in the presence of MA or AMA the TMR group was very quickly degraded. The TMR-group is stable, however, using *tert*-butylamine (TBA) / water (1:3) or TBA / MeOH / water (1:1:2) at elevated temperatures.[142]. However, it was found that the exocyclic amine acetyl base protecting groups were incompletely removed by this method of deprotection. Repeated attempts at temperatures up to 85 °C and times up to several days were undertaken without success. MALDI-TOF analysis clearly showed the remaining acetyl group peaks of RNA **6** (Figure 11). The 5′-TMR-mir-19b-2 fragment **6** was then purchased and used in the subsequent formation of the beacon.

5′-TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′ **(6)**

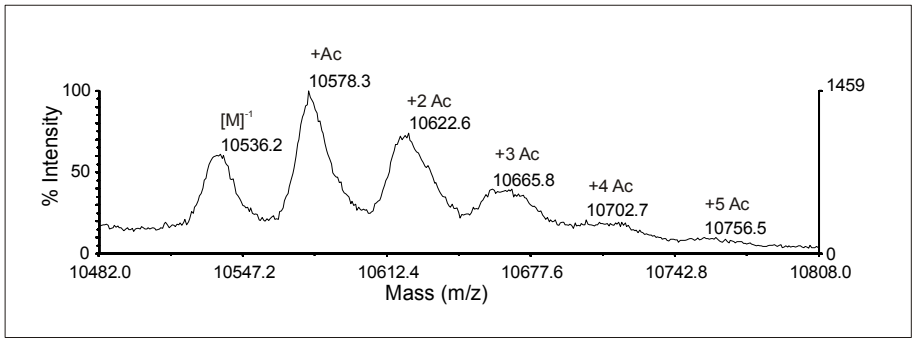


Figure 11: MALDI-TOF spectrum of **6** attempted using TOM protecting group chemistry. Up to 5 acetyl groups (+43 Da) are seen, which were unsuccessfully removed with TBA reagent.

In order to overcome such problems with TMR or any other sensitive modifications a common method is to synthesize the RNA containing an amine or other reactive group. The desired modification is then introduced after deprotection of the oligonucleotide, such as by using an *N*-hydroxysuccinimide ester with an amine. This is very common, but somewhat inefficient, since additional steps are required (purification steps included), leading to a decreased yield of the final product.

Another RNA synthesis option is to use the phenoxyacetyl (A) or *p*-isopropylphenoxyacetyl (G) amine protecting groups. Combined with acetyl-protected C these bases can be deprotected using so-called ultra mild conditions (commercially available as 2′-TBDMS chemistry). They most certainly could be deprotected with TBA reagent, however, for general usability the universal support (USIIPS) would be preferable over standard RNA cpg. Using ultra mild conditions the RNA is cleaved from cpg and exocyclic bases and phosphate protecting groups are removed in the presence of only 0.05 M potassium carbonate in MeOH.

Additionally, the use of the BMT activator significantly reduces the coupling time with 2′-TBDMS protected phosphoramidites.[143] Thus, the perceived advantages of commercially available TOM-chemistry (no 2′-protecting group migration, better yields) must be weighed against any disadvantages (lack of compatibility with sensitive modifications, expense). Particularly in respect to yield, the TOM chemistry might not owe its greater yields to a less hindering 2′-protecting group, but to the simple use of the better activator BMT.[143]

The full length beacons might also be synthesized completely on cpg, except that yields are known to be significantly poorer for RNA as compared to DNA. Purification could also be

problematic owing to any failure sequences. Even with the use of BMT as activator the synthesis of long RNA oligonucleotides still remains a problem.[144] Another possibility could be through the use of ACE chemistry (bis(2-acetoxyethoxy)-methyl orthoester).[145]

3.1.2 Beacon Formation *via* Ligation with T4 RNA Ligase

With the synthetic RNA strands at hand beacon formation was attempted *via* ligation with T4 RNA ligase. It is known that a 3′-hydroxy acceptor RNA can be ligated with a 5′-phosphate donor RNA to create the full length product. An additional phosphate group on the 5′-terminus of the donor prohibits self-ligation.[146, 147] In this case the inherent complementarity of the product should prohibit any self-ligation. Indeed, the synthetic RNA containing a 5′-fluorophore and 3′-hydroxyl group could be successfully ligated with RNA containing a 3′-DABCYL group and 5′-phosphate in the presence of T4 RNA ligase using the manufacturer's conditions. Using this method yields were generally about 30-40%, but the formation of the pre-let-7 beacon **10** as well as its FAM-only version **11** reached yields of 70%.

A 5′-adenylated intermediate of the 3′-DABCYL RNA was also observed in most cases. This is a known side reaction of ligation with T4 RNA ligase and can be suppressed somewhat by performing the ligation reaction at a pH of 8.3.[146] Greater yields could perhaps also be achieved by the use of longer reaction times at lower temperatures.[148, 149] It might also be possible to achieve selective ligation in the presence of DMSO. Logic would dictate that hybridization of the 5′- and 3′-strands should promote ligation owing to the spatial proximity of the free termini, however, it might just be desirable to prevent hybridization of the strands with each other to make the position more accessible to the enzyme.[148, 149]

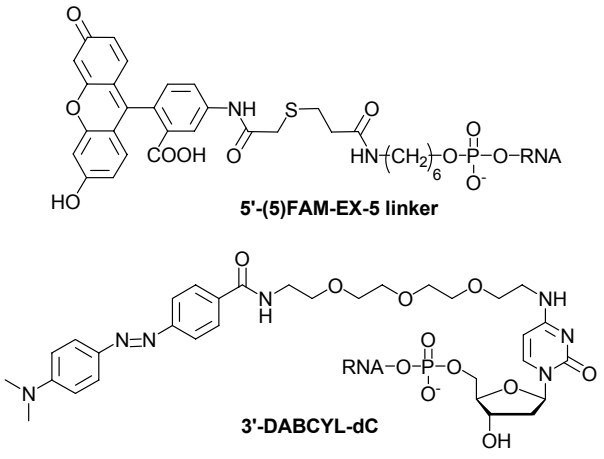


Figure 12: 5′-FAM and 3′-DABCYL dyes used in pre-let-7 beacon **10** and **11**.

For the pre-let-7 beacon the 5′-FAM 36 mer and 3′-DABCYL 37 mer strands were purchased from IBA (Figure 12 shows modifications). The high yield of the ligation reaction for the pre-let-7 beacon **10** (70%) can be seen by native PAGE analysis as the educt strands are hardly visible after ligation (Figure 13, gel B / C, lane 4). The beacon is already highly quenched without prior renaturation (gel B, lane 4). This is strong evidence of hairpin formation. Also, even the simple mixture of the two beacons without renaturing leads to some quenching of fluorescence (gel B, lane 3). The efficiency of DABCYL quenching can be observed in gel C. Upon staining with SYBR Green II the DABCYL strand is barely visible (gel C, lane 2), while the ligation product can now be seen (gel C, lane 4). This quenching, even in the presence of the SYBR stain underscores the effective quenching of the DABCYL group.

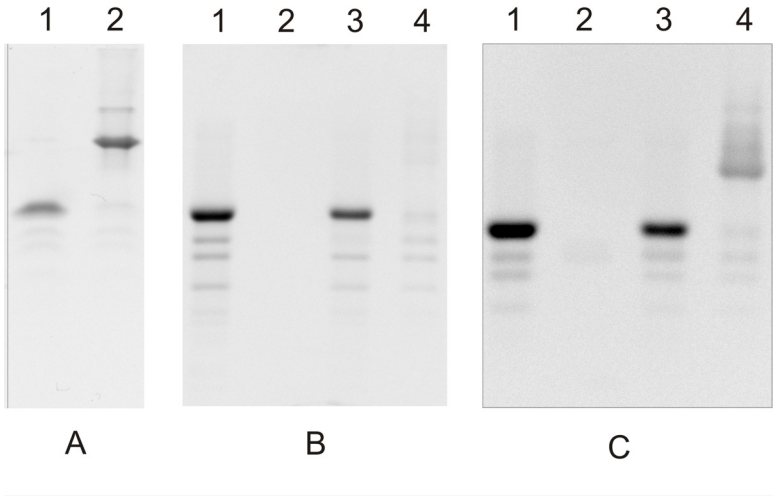


Figure 13: A) Denaturing PAGE of ligation reaction of pre-let-7 beacon **10**: Fragments mixed (lane 1), ligation product (lane 2). B) Native PAGE of ligation without staining: FAM 36 mer (lane 1); DABCYL 37 mer (lane 2), educt strands mixed (lane 3), ligation product (lane 4). C) Same as in B except stained with SYBR Green II. All gels were 20%.

Purification was done using RP-HPLC, the lipophilicity of the DABCYL group in all cases allowing good separation of the products from the educt strands as well as any degradation by-products. Pure product was obtained as shown by MALDI-TOF analysis (Figure 14). Worries of RNAse degradation *via* normal RP-HPLC were allayed, at least for the ligated beacon product as this was found to be stable for up to several years at -20 °C with hardly any degradation as seen by MALDI-TOF and PAGE analysis (data not shown).

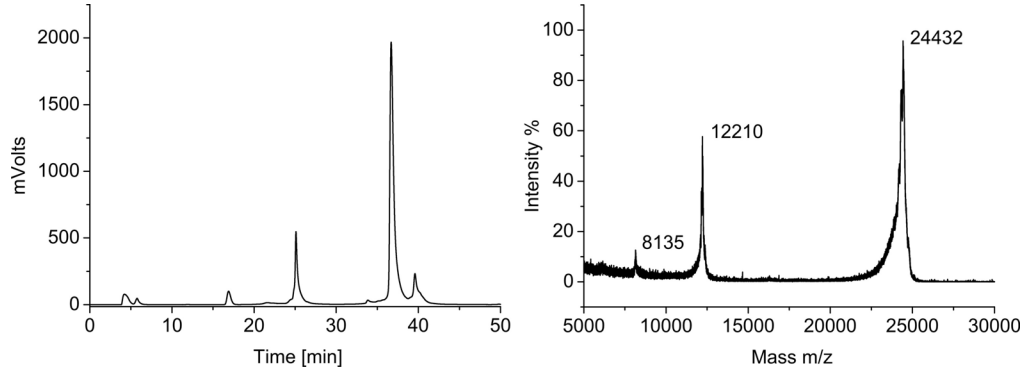


Figure 14: HPLC chromatogram of purification, t_R = 37 min. (left) and MALDI-TOF spectrum of pre-let-7 beacon **10** calc. m/z 24411 $[M-H]^{1-}$, 12205 $[M-H]^{2-}$, 8136 $[M-H]^{3-}$. Found: 24432, 12210, and 8135.

Melting curve analysis of the purified pre-let-7 beacon **10** showed sigmoidal behavior as further evidence of the hybridized hairpin structure. The sample was first taken up in buffer (20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) and renatured before measuring. The first derivative gave a T_m = 59 °C.

For the 66 mer TMR pre-mir-19b-2 beacon **11** the 5′-TMR strand **6** was successfully ligated to its 3′-partner strand **7**. However, for use in the assay (see section 3.4) the beacon was made using the 5′-TMR strand **6** purchased from IBA. A total of eight beacons were synthesized using the ligation method (Figure 15), which allowed easy purification by standard RP-HPLC in all cases.

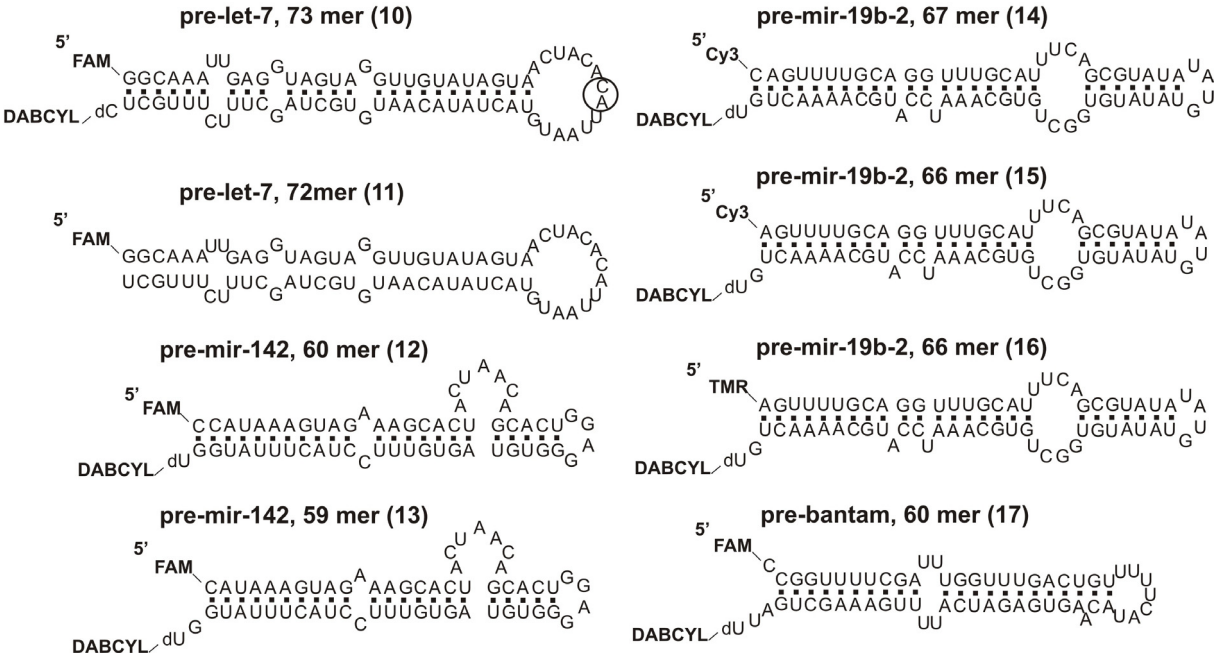


Figure 15: Chemically synthesized beacons formed *via* ligation. Typical ligation position is noted by a circle. Secondary structures shown as calculated with RNAstructure 4.5.[133]

3.2 Assay Development

The next step was to assure that the miRNA maturation probes could be processed by the Dicer enzyme. Following the manufacturer's guidelines incubation of the pre-let-7 beacon **10** with commercial recombinant human Dicer (rhDicer) showed a Dicer-mediated fluorescence increase over the course of 18 hours (Figure 16). Although Dicer is known to be quite inefficient, this rate of cleavage was in agreement with the manufacturer's specifications.[35, 37, 150] In contrast, fluorescence of the pre-let-7 RNA **11** containing no 3′-DABCYL group is hardly quenched and only a minimal fluorescence increase could be observed (data not shown, see also section 3.5).

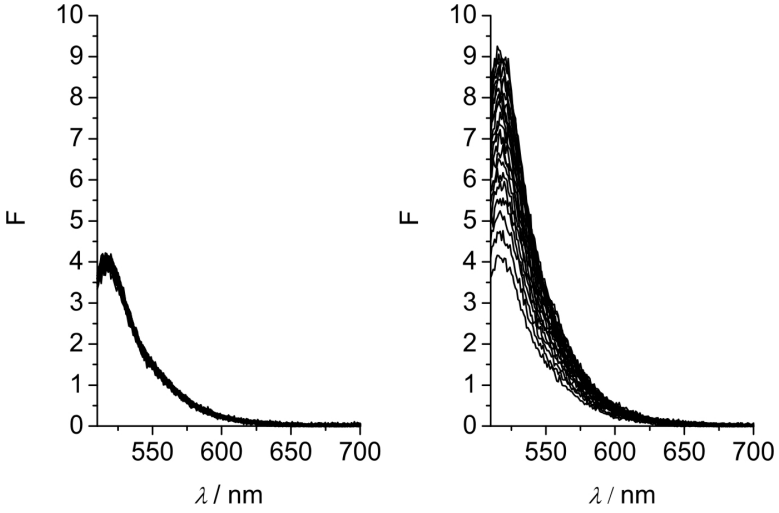


Figure 16: Incubation of 700 nM pre-let-7 beacon **10** with heat-denatured rhDicer (left) compared to that with rhDicer (Stratagene) (right). Conditions: 25 U Dicer, 20 mM Tris-HCl, pH 7.4, 250 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 1 mL. Measurements were taken every hour for 18 h with ex = 491 nm and slit widths ex = 5 nm and em = 2.5 nm.

In its natural context Dicer interacts with various proteins such as the transactivating response binding protein (TRBP), which increases Dicer-mediated cleavage *in vitro* and is required *in vivo* for proper functioning of the enzyme.[28] Furthermore, a specific pre-miRNA binder that inhibits Dicer cleavage should still lead to a reduced fluorescence signal in the presence of other RNAs. Accordingly, incubation of beacon with cell lysate from HEK 293 cells lead to a faster and overall greater fluorescence signal increase (Figure 17). In addition, only 20 nM beacon could be used compared to 700 nM with the recombinant enzyme.

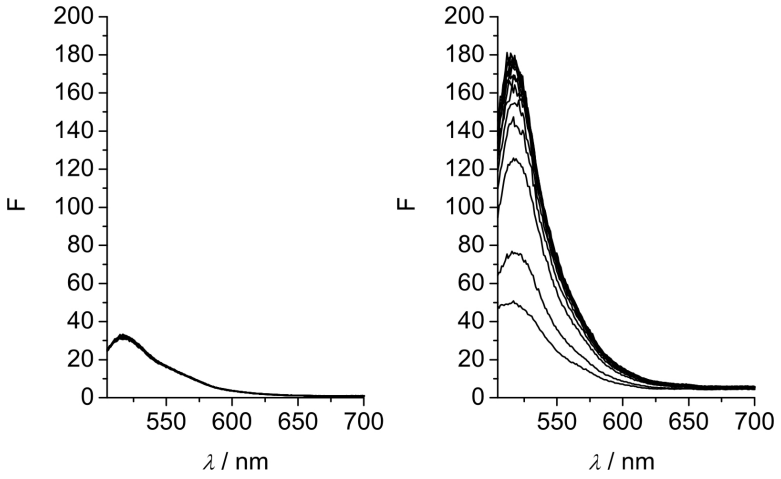


Figure 17: Fluorescence increase upon incubation of 20 nM pre-let-7 beacon **10** with 10% heat-denatured HEK 293 lysate (left) compared to that with 10% HEK 293 lysate (right). Further conditions: 20 mM Tris-HCl, pH 7.4, 75 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 1.1 mL. Measurements were taken every 30 min. for 6 h with ex = 475 nm and slit widths ex = 20 nm and em = 10 nm.

To eliminate the possibility of RNAse contamination in the cell lysate the reaction was also tested in the presence of RNAse inhibitor. The assay was now also tested in 96-well format using a plate reader to detect fluorescence. Nearly identical fluorescence curves were obtained as shown in Figure 18. The selectivity of the RNAse inhibitor may be explained by the fact that Dicer belongs to the type III family of RNAses, which is distinct from the type I family of RNAses such as RNAse A.

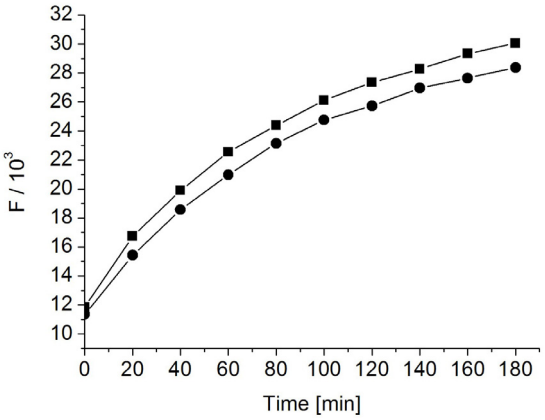


Figure 18: Fluorescence increase upon incubation of 50 nM pre-let-7 beacon **10** with (●) and without (■) 40 U RNAse inhibitor in the presence of 10% cell lysate. Further conditions: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$ in 100 µL in a 96-well plate.

The optimal buffer conditions for commercial rhDicer included significantly lower NaCl concentrations than those recommended by the manufacturers as is known from the literature.[35, 150] Various NaCl concentrations as well as the presence of DTT were also tested (Figure 19). Optimal conditions for the assay included 20 mM Tris-HCl, pH 6.8, 12 mM NaCl, 1 mM DTT, and as little as 10 nM beacon in 40 µL in a 384-well plate. A standard concentration of 20 nM beacon was selected as the working concentration.The cost of the assay based on the beacon was calculated to be only a few Euro cents per well (96-well format). For high throughput screening of libraries of potential miRNA binders a 1536-well plate can also be envisioned. It is interesting to note in Figure 19 that in fact at 250 mM NaCl without DTT no fluorescence increase is seen. Only with a much higher beacon and enzyme concentration is cleavage observed at this high salt concentration (see Figure 16 above).

Slight differences could also be observed with rhDicer from different commercial sources. It was found, much as described by Vermeulen *et al*, that the different commercial Dicer enzyme preparations delivered somewhat different activities.[46] Dicer preparations from Stratagene, Genlantis (Gene Therapy Systems) and Ambion all performed similarly (all containing ~10% full length enzyme). It was also found that the best commercial Dicer preparation was that from Invitrogen. Together with the optimized buffer described above, the Invitrogen Dicer can be diluted up to 40x in a separate buffer containing 0.1% Triton X-100 [35] and as little as 0.1 U enzyme in a 40 µL reaction volume can be used. Recombinant human Dicer has since been successfully overexpressed from Sf21 insect cells in the Arenz group to ensure a steady supply of the enzyme for the future.[151]

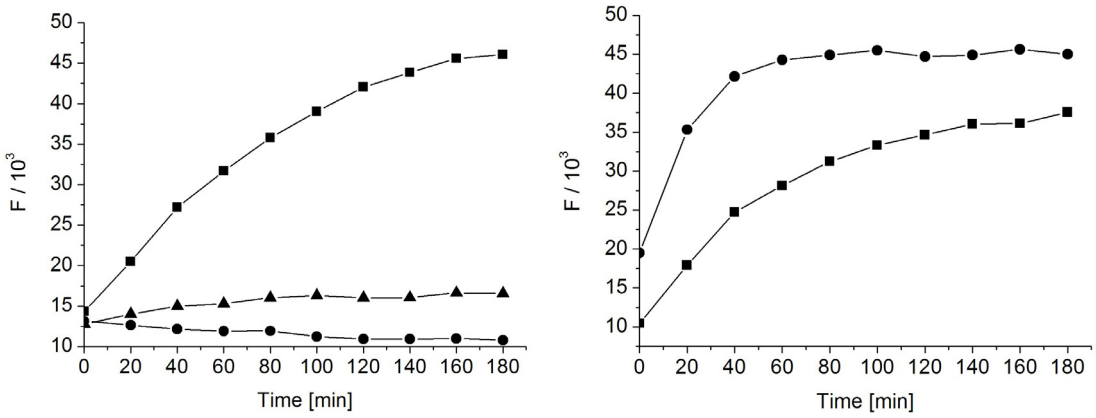


Figure 19: Fluorescence increase upon incubation of pre-let-7 beacon **10** with rhDicer. Left: 50 nM beacon, 1 U Dicer (Stratagene) and either 250 mM NaCl (●), 150 mM NaCl (▲), or 150 mM NaCl + 1 mM DTT (■) in 100 µL in a 96-well plate. Right: 10 nM beacon with 0.25 U Dicer (Genlantis), 1 mM DTT and either 75 mM NaCl (■) or 12 mM NaCl (●) in 40 µL in a 384-well plate. Further conditions: 20 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$.

Analysis of Dicer cleavage products of the pre-let-7 beacon **10** and the homologous *in vitro* transcript were compared *via* denaturing PAGE. The pre-let-7 beacon **10** contains a 3′-overhang of a single nucleotide if the deoxyuridine-DABCYL moiety is taken into account. This structure was initially chosen as it should more easily lead to release of the 5′-FAM section from the complementary 3′-DABCYL-containing section, thus better assuring a fluorescence increase. This precaution was later found to be unnecessary (see section 3.4). The *in vitro* transcript also contains such a structure (3′-C/U overhang). This type of structure should result in the typical 21-23 mer cleavage products produced by Dicer, but also a longer ~26 mer. From these longer structures it is known that a subsequent cleavage step releases a 21-22 mer along with the remaining 4-5 mer.[46] Indeed, both the longer and shorter structures can be seen in the gel (Figure 20, lane 3 and 6, single arrows). With the beacon digest (lane 3) the shorter FAM-containing strand can also be observed (double arrow).

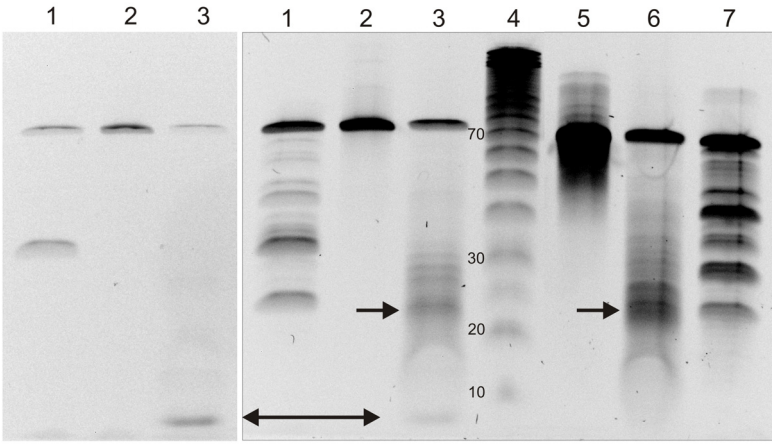


Figure 20: Denaturing 15% PAGE gel without (left) and with (right) SYBR Gold staining showing Dicer cleavage of both pre-let-7 beacon **10** (lane 3) and the homologous *in vitro* transcript (lane 6) with controls (lanes 2 and 5, respectively). The expected bands corresponding to 22-23 mers can be seen (single arrows). Just above this can be seen bands in the 25-26 mer range. The double arrow points out the short FAM fragment. Lanes 1 and 7 show T1 RNAse digests of the beacon and transcript, respectively. Lane 4 is an RNA size marker with pertinent sizes labeled. Beacon (0.25 µg) or transcript (1µg) was incubated in Dicer buffer with 4 U RNAse inhibitor and 0.5 U rhDicer (Genlantis) in a total volume of 15 µL for 6 hours at 37 °C.

For comparison, the T1 RNAse digest of the beacon releases the 30 mer FAM-containing fragment (calc. $[M-H]^{1-}$= 10474.0, found 10470.2), which was also visible under light excitation before the gel was stained with SYBR Gold (Figure 20, left). After staining, 25 mer sequences corresponding to two different sections could then be seen just below the longer section (calc. $[M-H]^{1-}$= 7941.0, found 7941.2).

With the beacon the short secondary cleavage fragment 4-5 mer can also be observed in this gel picture. Bands corresponding to Dicer primary cleavage of 21-24 mers directly from the 5′-terminus can only be made out weakly. Also only lightly fluorescent are bands corresponding to primary 25-26 mer FAM-containing segments. These would be quickly processed further to the corresponding 22-33 mers, which appear after staining with SYBR Gold.

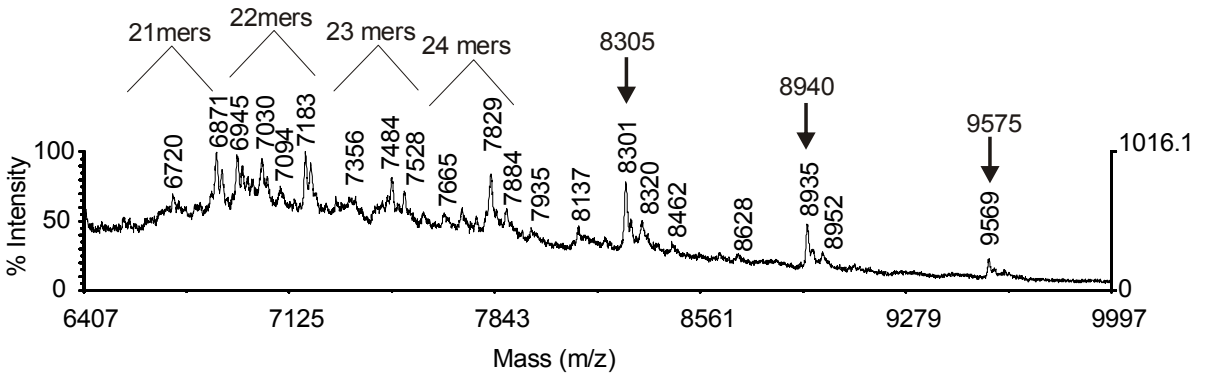


Figure 21: MALDI-TOF analysis of 21-26 mer bands eluted from PAGE gel after Dicer cleavage of pre-let-7 beacon **10**. 21-24 mer fragments are marked to the left. Calculated masses are designated above the respective measured peaks (right).

The bands in the range of 21-26 were excised, eluted, and alcohol precipitated (NH_4OAc, ammonium acetate). MALDI-TOF was then used to analyze the masses resulting from Dicer cleavage. The most pronounced peaks are those pertaining to 22 mers (calc. $[M-H]^{1-}$= 7187, found 7183) resulting from apparent secondary cleavage from longer 24 / 26 mers. Peaks pertaining to hairpin sections can be made out pertaining to primary cleavage of 22-26 mers directly from the 5′-terminus (Figure 21; calc. $[M-H]^{1-}$= 9575, 8940, 8305 labeled, and 7670 and 7034 unlabeled). Additional masses pertaining to secondary cleavage of 21 and 23 mers are also present. The short 4-5 FAM-containing fragment could not be satisfactorily identified *via* MALDI-TOF analysis. Therefore, HPLC-ESI (Agilent 1200 with ESI, Polaris column) was used in positive mode to measure the mass of the fragments. Only the fragments 5′-FAM-GGCA (calc. $[M-H]^{1+}$= 1918.4, found 1913.9) and 5′-PO_3-CU-DABCYL-3′ (calc. $[M-H]^{1+}$= 1345.6, found 1337.8) could be identified (Figure 22). These fragments would result from primary cleavage of a 26 mer from the 5′-terminus with secondary cleavage of a 22 mer from this segment.

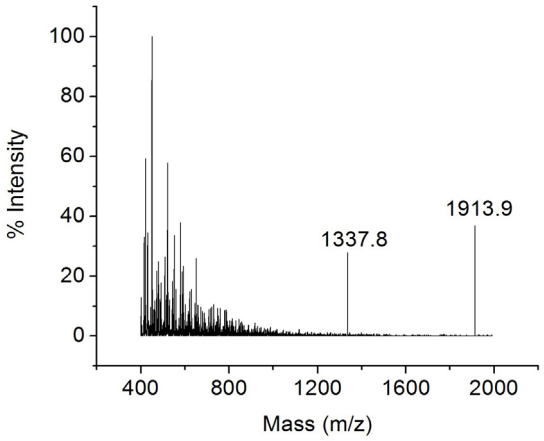


Figure 22: HPLC-ESI measurement (positive modus) of FAM-fragment of pre-let7-beacon **10** shows two masses most likely corresponding to those of the 5′-FAM- GGCA (1913.9) and 5′-PO_3-CU-DABCYL-3′ (1337.8) fragments.

3.3 Inhibitors of miRNA Maturation

Having established that Dicer is able to cleave the beacon efficiently, the next step was to see if the homogenous fluorescent assay could be used to detect inhibitors of miRNA maturation. Of utmost importance here is that any substances bind the target pre-miRNA and not Dicer itself, since this mechanism of inhibition could be deadly for an organism.[152, 153] The known RNA binder kanamycin A [85] was tested at 100 µM and found to inhibit cleavage of the pre-let-7 beacon **10** by 69 ± 3% over 2 hours (Figure 23).[154]

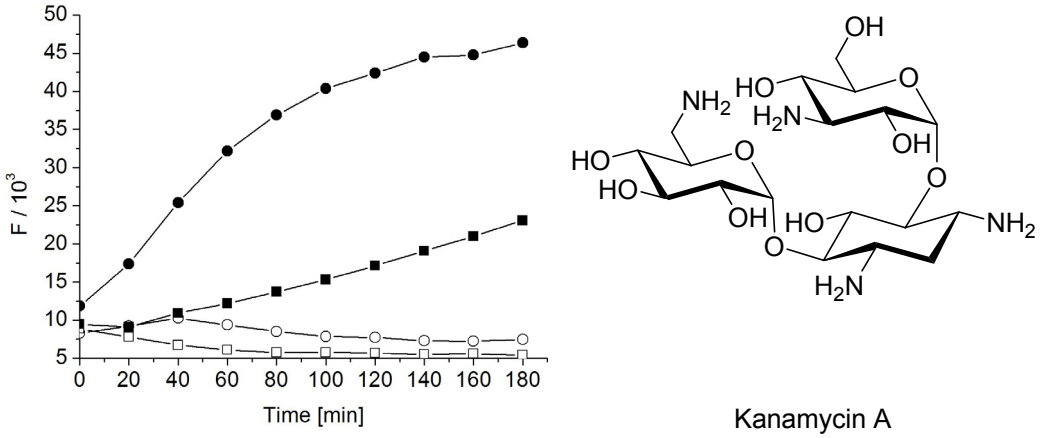


Figure 23: Fluorescence increase upon incubation of 0.5 U rhDicer (Genlantis) with 20 nM pre-let-7 beacon **10** alone (●) or in the presence of 100 µM kanamycin A (■). Controls: heat denatured rhDicer with (□) or without (○) 100 µM kanamycin A. Further conditions: 20 mM Tris-HCl pH 7.4, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 40 µL in a 384-well plate.

Since Dicer inherently binds pre-miRNA, short dodecapeptides derived from the Dicer peptide sequence were also tested as miRNA maturation inhibitors. Various sequences were pre-selected using a peptide scan.[155, 156] The most promising sequences were then synthesized on solid phase [157] and tested against the pre-let-7 beacon **10** (Figure 24). At a concentration of 100 µM peptide **19** showed an inhibition of 82 ± 4% over two hours (Table 2). For comparison, at the same concentration peptide **18** and peptide **20** showed inhibition rates of 0 ± 4% and 4%, respectively, over two hours.

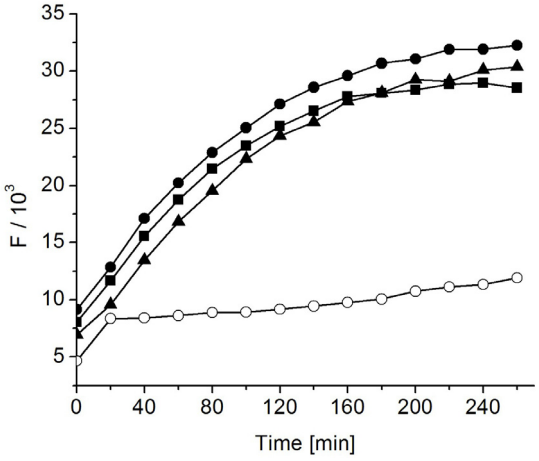


Figure 24: Fluorescence increase upon incubation of 0.5 U rhDicer (Genlantis) with 30 nM pre-let-7 beacon **10** alone (●) or in the presence of 100 µM peptide **18** (▲), **19** (○), or **20** (■). Further conditions: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 100 µL in a 96-well plate.

Table 2: Inhibition of let-7 miRNA maturation by Dicer-derived dodecapeptides at 100 µM. The synthesized peptides sequences are inverted with respect to the original Dicer sequence.

Dicer Peptide	Inhibition (%)
^{Ac}NH-SSIYALEPDQKG-$CONH_2$ **18**	0 ± 4
^{Ac}NH-SSIYALEPDQKG-$CONH_2$ **19**	82 ± 4
^{Ac}NH-RYNIKKEFNEFG-$CONH_2$ **20**	4

As controls 10 µM $ErCl_3$ and denatured rhDicer were measured together with peptide **19** in a separate experiment (Figure 25).[158, 159] $ErCl_3$ is known to bind the active site of Dicer.[40]

No fluorescence increase is seen in the presence of $ErCl_3$, while the inhibition by peptide **19** remained the same within experimental error (77 ± 1%). Another control would be an antibody against Dicer, however, incubation with Dicer antibody (Ambion) did not lead to an inhibition of fluorescence increase either with the cell lysate or with the purified recombinant enzyme (data not shown). This points to binding by the antibody of an epitope that is not responsible for the catalytic activity of the enzyme.

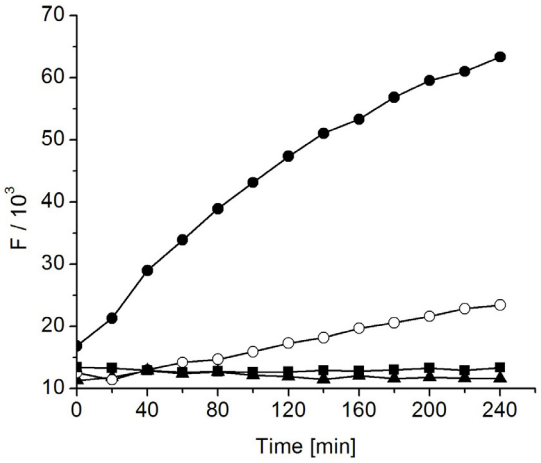


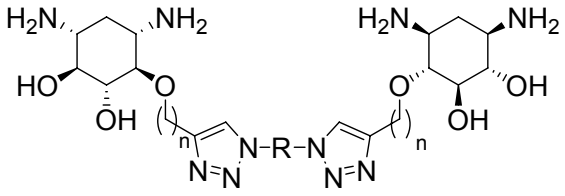
Figure 25: Fluorescence increase upon incubation of 0.5 U Dicer (Genlantis) with 20 nM pre-let-7 beacon **10** with (○) or without (●) 100 µM peptide **19**. Controls: 10 µM $ErCl_3$ (■) or heat denatured Dicer plus 100 µM peptide **19** (▲). Further conditions: 20 mM Tris-HCl pH 7.4, 75 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 100 µL in a 384-well plate.

Various other peptides were also tested, including cyclic versions of several of the most strongly binding peptides, however none of the variants tested showed as great an inhibition as peptide **19**. The binding affinities of several of these peptides to pre-let-7 beacon **10** were then determined using surface plasmon resonance (SPR, 'Biacore'). The K_D of peptide **19** was approximately 50 µM.[157]

These results were promising, but further validation of the assay was needed in order to rule out inhibition through Dicer binding or binding to the fluorophore or quencher moieties, which would lead to false positive results. A small library of compounds was synthesized and tested amongst the various beacons and the results compared to SPR binding data of the non-fluorescent pre-miRNA.

A small collection of aminoglycoside analogues based on 2-deoxystreptamine (2-DOS) was tested as potential miRNA maturation inhibitors. The compounds were synthesized in the

laboratory [160] using the Sharpless-Meldal variant of the Huisgen 1,3 dipolar cycloaddition.[161] This so-called 'click chemistry' is widely applied to conjugation of azides to terminal alkynes *via* the formation of triazoles (Figure 26). The synthesis was based on work by Hergenrother and coworkers.[111] This method was chosen for its apparent ease and the fact that similar compounds had already been shown to be selective and high-affinity binders of artificial hairpin RNA.



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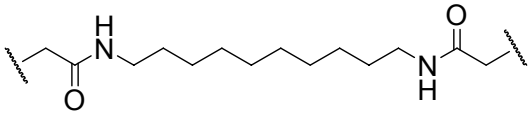
21	1	
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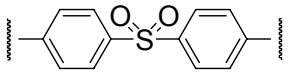
23	1	
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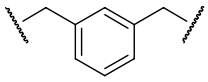
25	1	
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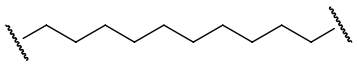
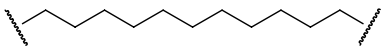
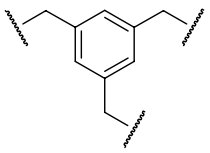
27	3	
28	3	
29	3	

Figure 26: Various 2-DOS conjugates tested.

At a concentration of 100 µM the compounds showed varying inhibition rates of Dicer processing amongst the selection of beacons (Figure 27). The three conjugates **23**, **24**, and **29** showed nearly complete inhibition with all beacons at this concentration. Compounds **26** and **28** also inhibited the cleavage of beacons pre-bantam **17** and pre-let-7 **10** nearly fully. Amongst these four beacons some selectivity can also be seen, such as with compounds **25**, **26** and **27**.

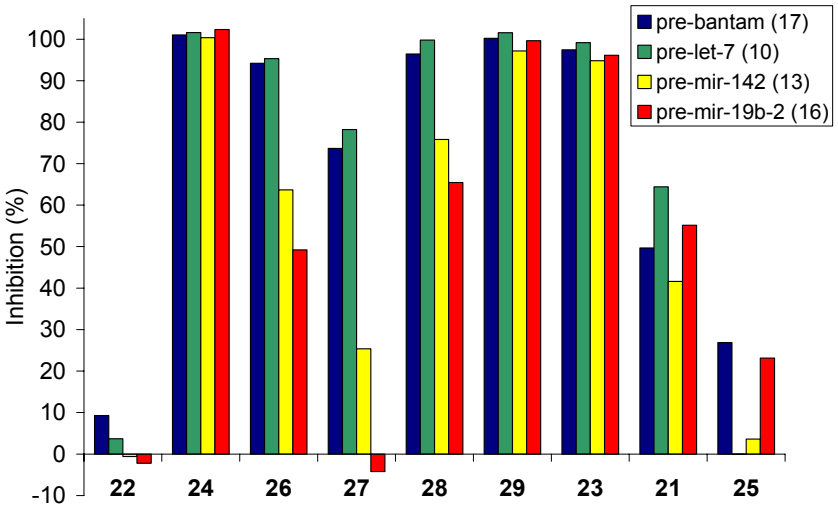


Figure 27: Inhibition of Dicer cleavage by 2-DOS conjugates at 100 µM. Inhibition (%) was measured between 20-120 min. under optimized buffer conditions. Results are the averages of two measurements. The standard error of the mean is less than 5% for all measurements.

The best of these inhibitors were then tested at a concentration of 25 µM (Figure 28). Two compounds, **24** and **29**, still inhibit nearly 100% with the pre-bantam **17** and pre-let-7 **10** beacons. Also here a slight selectivity is seen with compound **23** for the three beacons compared to the mir-19b-2 beacon **16**.

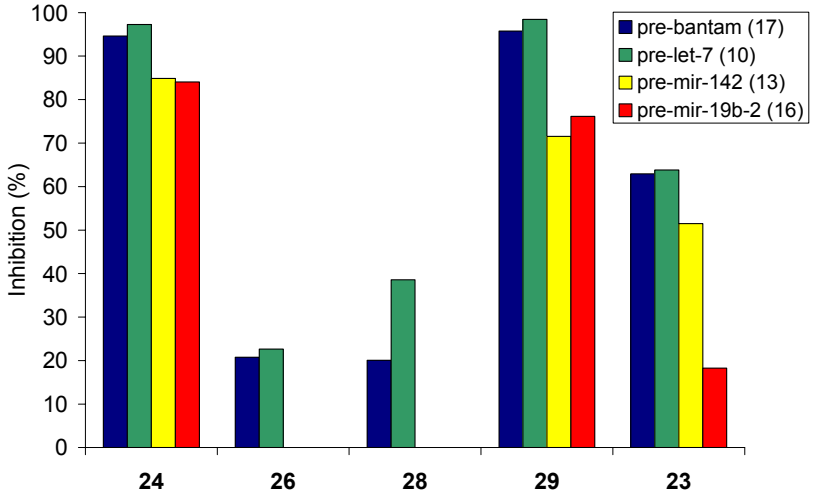


Figure 28: Inhibition of Dicer cleavage by 2-DOS conjugates at 25 µM. Substances **26** and **28** were not tested at this concentration with pre-mir-142 (**13**) or pre-19b-2 (**16**) beacons since only minimal inhibition was observed at 100 µM. Inhibition (%) was measured between 20-120 min. under optimized buffer conditions. Results are the averages of two measurements. The standard error of the mean is less than 5% for all measurements.

The IC_{50} values of the best inhibitors were then determined. For the pre-let-7 beacon **10** K_D were also calculated from kinetic analysis of surface plasmon resonance (SPR) data (Table 3). For SPR measurements the homologous 5′-biotin-labeled pre-let-7 obtained by *in vitro* transcription was bound to the chip *via* streptavidin. For pre-let-7 beacon **10** a very good correlation between inhibition of the Dicer processing of pre-let-7 beacon and binding of the molecules to the RNA can be seen. This is evidence of binding of the RNA, which leads to cleavage inhibition.

Table 3: IC_{50} values for pre-bantam **17** calculated from assay as well as IC_{50} values and corresponding K_D values from SPR for pre-let-7 beacon **10**. Inhibition values for IC_{50} calculation were measured in triplicate. Error values represent the error of the least squares calculation. See Supplementary for graphs.

Compound	**pre-bantam**		**pre-let-7**	
	IC_{50} (µM)		**IC_{50} (µM)**	**K_D (µM)***
26	-		37.9 ± 0.1	2.0
28	-		32.3 ± 0.2	1.9
24	13.9 ± 0.5		12.8 ± 0.3	1.0
29	6.0 ± 0.7		8.1 ± 1.1	0.4

*Measurements performed by Saskia Neubacher, AK Arenz, Humboldt-Universität zu Berlin.

Although the binding and inhibition data correspond well with one another in these cases, it is possible that binding to the target RNA is necessary, but not sufficient for cleavage inhibition, since pre-miRNAs very often contain multiple potential binding epitopes in the form of bulges, unpaired bases, and loops. A given compound might bind the terminal loop very well, for example, where Dicer might still be capable of processing the RNA. Another compound might bind Dicer preferentially and not the RNA at all.

Additionally, the results presented here show only little selectivity among the various pre-miRNA beacons. A drug candidate must be capable of specifically binding a desired pre-miRNA amongst a complex mixture of RNA in the cell. In order to study such interactions an assay is needed where multiple beacons containing different fluorogenic groups are present together in the same reaction mixture in order to detect selective inhibitors of miRNA

maturation. Finding a specific binder of a single pre-RNA beacon using such a multiplex assay would be a first step towards development of selective pre-miRNA binders.

The simplest case of such an assay would use two beacons, each with a different fluorogenic group (duplex assay). Ideally, in the presence of a selective RNA binder, the fluorescence from the one fluorophore would increase as expected (the inhibitor does not inhibit cleavage) whereas the fluorescence from the other fluorophore remains on the baseline (the inhibitor inhibits cleavage) (Figure 29). At the same time such a differentiated fluorescence increase would be further evidence that the inhibition is the result of the RNA being bound and not Dicer.

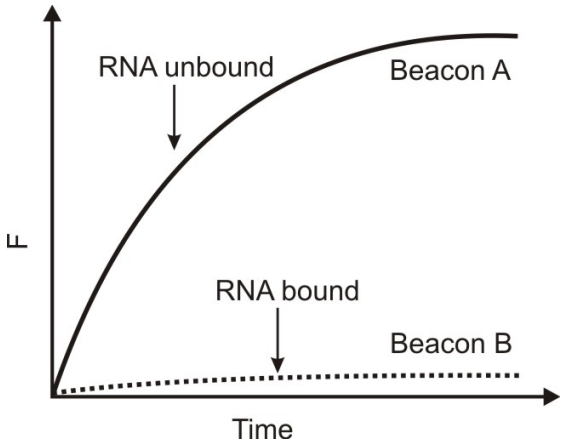


Figure 29: Theoretical example of duplex assay. Inhibitor binds selectively beacon B leading to no fluorescence increase. Unbound beacon A cleaved by Dicer leads to fluorescence increase.

3.4 Duplex Assay

A duplex assay containing two differently labeled and unique pre-miRNA beacons was the first step towards being able to detect selective pre-miRNA binders. It was desirable though, to first have a beacon that more closely resembled the native structure containing a two nucleotide 3′- overhang. To this end two sets of beacons were synthesized containing either FAM or Cy3 as the fluorogenic group and either a 1- nt or 2- nt 3′-overhang. Two human pre-miRNAs were selected based on their differing calculated secondary structural elements as well as their opposing expression levels in cancer.

Mir-142 has been shown to be underexpressed in various types of cancer.[162] It has been suggested that a translocation of the *c-myc* gene into the mir-142 locus leads to disruption of the mir-142 expression and thus overexpression of the c-Myc protein (an important

transcription factor), leading to uncontrolled cell growth.[163, 164] In contrast, mir-19b-2 belongs to the mir-17-92 cluster of miRNAs and has been shown to be overexpressed in various lymphomas [68] and lung cancer.[69] c-Myc activates expression of this cluster, which leads to enhanced angiogenesis.[165] The mir-17-92 cluster is thought to act as an oncogene.[166]

The FAM-labeled pre-mir-142 containing a 2-nt 3′-overhang **13** was shown to be processed the same as its 1-nt overhang homolog **12** leading to a 4-fold fluorescence increase over the course of 4 hours (Figure 30). The pre-mir-19b-2 was chosen for formation of the second beacon with Cy3 as fluorophore. As can be seen, both beacons were also processed by Dicer to the same extent. Unfortunately, the fluorescence only increased by 0.3-fold over the course of 4 hours compared to the 3.6-fold (± 0.4) and 4.2-fold (± 0.2) increases seen with the FAM-labeled mir-142 structures (59 mer **13** and 60 mer **12**, respectively). Additionally, a significant increase could only be seen well with 100 nM Cy3-labeled beacon in the presence of 0.05% Tween 20. The low fluorescence could be due to the low quantum yield of Cy3 (Cy3 0.1, FAM 0.9) [167, 168] or to its more lipophilic nature compared to the FAM group (own observations). Some precipitate was observed with the Cy3 beacons after Dicer processing, which could mean a loss in fluorescence due to aggregation in the aqueous buffer. The Cy3-labeled beacons, however, would show their usefulness in the cellular assay (see section 3.6).

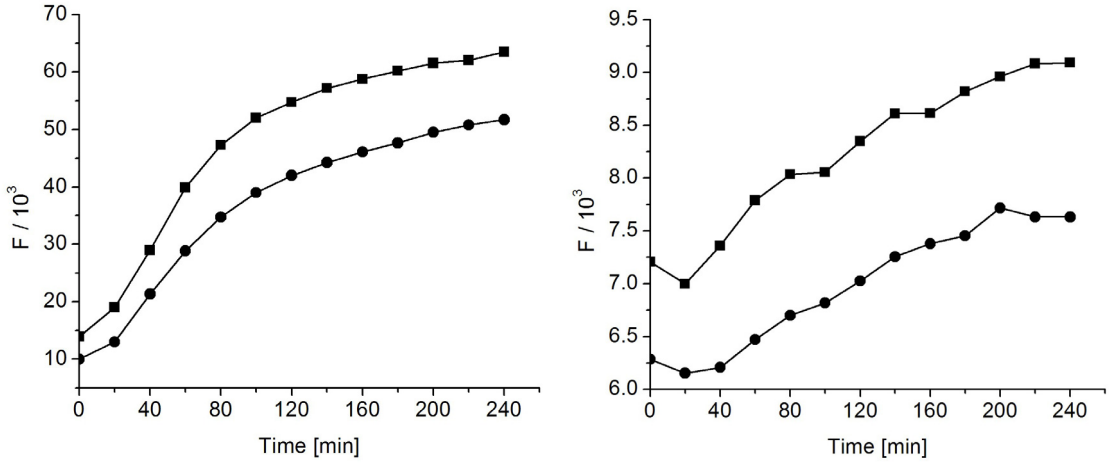


Figure 30: Incubation of 1 U Dicer (Ambion) with beacon in 40 µL. Left: 20 nM FAM pre-mir-142 beacon **13** (■, 59 mer) or **12** (●, 60 mer). Right: 100 nM Cy3 pre-mir-19b-2 beacon **15** (■, 66 mer) or **14** (●, 67 mer) with 0.05% Tween 20. Further conditions: 20 mM Tris-HCl pH 6.8, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT.

The pre-mir-19b-2 beacon (66 mer, **16**) containing a 2′-nt 3′-overhang was then synthesized instead using 6-tetramethylrhodamine (TMR) as the 5′-fluorophore. TMR is very similar in structure to FAM and has a much higher quantum yield of 0.7.[167] The beacon could be ligated using the 5′-TMR strand **6**, which was incompletely acetyl deprotected, and the beacon was still able to be processed by Dicer, leading to a 0.7-fold fluorescence increase at 60 nM (data not shown). The same beacon made using the purchased 5′-TMR strand **6** showed a 1.7-fold (± 0.1) fluorescence increase at only 20 nM in the presence of rhDicer under the same buffer conditions (Figure 31). Although the self-made and purchased 5′-TMR strands **6** contained different linkers (C12 vs C6) and isomers (6 vs 5 isomer) the difference in processing efficiency by Dicer of pre-mir-19b-2 beacon **16** is most likely due to the incomplete acetyl-group deprotection. This emphasizes the importance of full deprotection of synthetic RNA for use in biological systems.

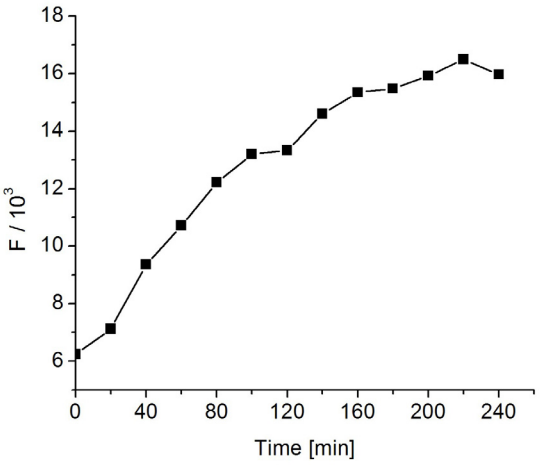


Figure 31: Incubation of 0.1 U Dicer (Invitrogen) with 20 nM TMR pre-mir-19b-2 beacon **16** in 20 mM Tris-HCl, pH 6.8, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, 0.001% Triton X-100.

Next, the both the FAM- and TMR-labeled beacons containing the 2-nt 3′-overhangs were tested together in the same reaction. Optimal reaction conditions included final concentrations of 60 nM beacon, 0.1% Triton X-100, and 1 U recombinant Dicer (Figure 32). This had the advantage of leading to a more uniform increase in the fluorescence of both beacons upon Dicer processing. The lower overall signal increase with TMR may be attributed to the poorer quenching by DABCYL,[169] as well as perhaps to the lower quantum yield compared to FAM, and the non-optimal excitation and emission filters available with the plate reader.

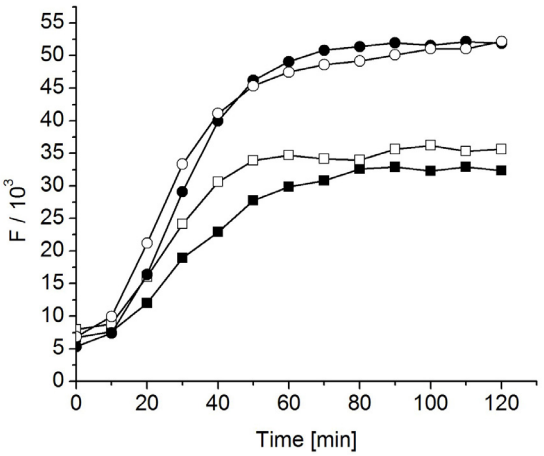


Figure 32: Duplex assay compared to individual measurements: FAM-mir-142 **13** alone (○) or in duplex format (●); TMR-mir-19b-2 **16** alone (□) or in duplex format (■). Optimized conditions included 60 nM beacon, 0.1% Triton X-100, and 1 U recombinant Dicer.

From earlier data of the various 2-DOS conjugates tested there were several candidates that showed the possibility of some selectivity between the two beacons here. Substances **21** and **23** and **25** were tested with the TMR- and FAM-labeled beacons in the duplex format (Table 4). The amount of inhibition of Dicer processing between the two beacons is compared in Table 4. Compound **21** at 100 µM shows identical inhibition with both beacons to within experimental error. This is in contrast to compound **23** at 50 µM were there was a more selective inhibition of the FAM beacon (58%) compared to the TMR beacon. And compound **25** at 100 µM showed a lack of inhibition of both beacons in the duplex format at 100 µM.

Table 4: Percent inhibition by various substances in duplex assay format. Calculation made between 10-40 min.

Compound	**Inhibition (%) with FAM Beacon 13**	**Inhibition (%) with TMR Beacon 16**
50 µM conjugate **23**	58 ± 3	-9 ± 3
100 µM conjugate **21**	38.0 ± 1.6	41 ± 1.9
100 µM conjugate **25**	6 ± 2	5 ± 2

These data show the first selectivity of miRNA maturation inhibition using the duplex assay format. Although the inhibitory concentrations are in the micromolar range this is a clear first step. Further evidence is needed to definitively rule out inhibition of the Dicer enzyme itself as opposed to inhibition through RNA binding, although the compounds tested are assumed to bind RNA instead of protein. Also, as it was desired to find binders and inhibitors of Dicer cleavage of the TMR mir-19b-2 beacon, which is overexpressed in cancer, further work is needed. Only the 2-DOS conjugate **23** showed some selective inhibition, however for the FAM pre-mir-142 beacon, which is *underexpressed* in cancer. A larger substance library is needed to find more specific inhibitors of miRNA maturation. Work is currently being done in the Arenz group towards this goal.

In a cell many more nucleic acids are present, let alone two pre-miRNAs. Using cell lysate as the Dicer source as well as multiple beacons would aid in the search for specific pre-miRNA binders. Several different pre-miRNA beacons with differing fluorogenic groups would be necessary. In addition to chemical synthesis of such pre-miRNA beacons as already described, *in vitro* transcription offers another route to synthesize the beacons, potentially in large amounts.[170]

3.5 Beacon Formation using *in vitro* Synthesis

The addition of modified groups to the 5′-terminus of nucleic acids by ‘priming’ an *in vitro* transcription reaction using 5′-modified ApG dinucleotides is well known.[171] Using T7 RNA polymerase it was possible to synthesize several beacons containing a 5′-fluorogenic group (Figure 33). The fluorogenic group was coupled *via* an NHS ester to the free amine of a C6-amino-linked 5′-ApG or introduced directly *via* a 5′-FAM-ApG or 5′-TMR-ApG transcription starter. The later method is preferred for the higher yields and simplicity, whereas the former method offers the flexibility of introducing different fluorophores onto the same sequence.

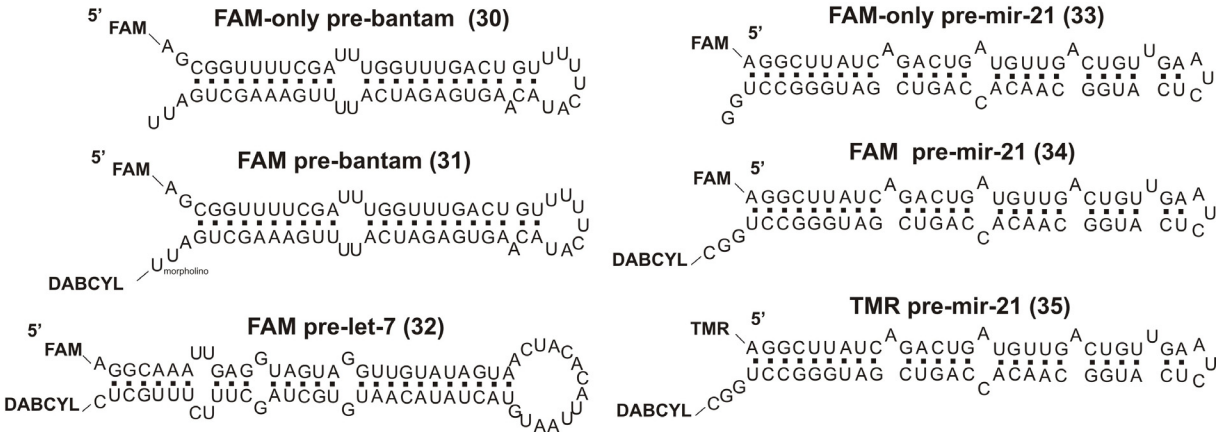


Figure 33: Beacons synthesized by *in vitro* transcription.

For attachment of the 3′-DABCYL group an attempt was first made using NHS ester coupling chemistry to attach DABCYL to an amino-ethylmorpholino derivative at the 3′-end.[172] Oxidative cleavage of the terminal 2′,3′-diol was carried out using sodium periodate. In the subsequent reductive amination in the presence of ethylene diamine a morpholino derivative could be synthesized containing a free ethylamino linker (Figure 34).

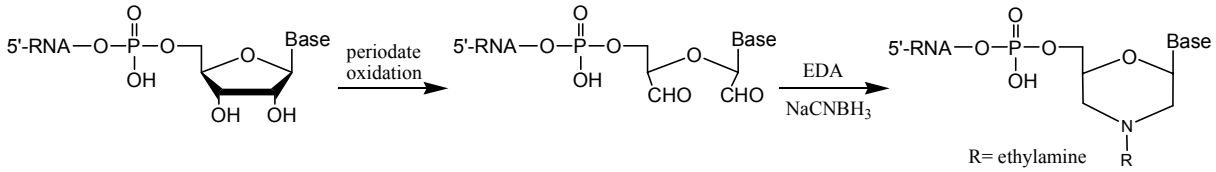


Figure 34: Modification of 3′-terminus of RNA for labeling with NHS esters.

After coupling of DABCYL *via* its NHS ester the final pre-bantam beacon **31** was found to be indeed processed by rhDicer (Figure 35). The FAM pre-mir-21 beacon **33** containing a GG 3′-overhang was also tested. It is known that guanosines close to a terminal fluorophore can quench its fluorescence.[168, 169] It was hoped that the DABCYL quencher could be done away with by creating just such an environment. Unfortunately, the quenching of such beacons was not great enough in these cases to be useful in the assay. Additionally, hardly any fluorescence increase was observed with the FAM-only pre-bantam beacon **30** upon incubation with rhDicer (data not shown).

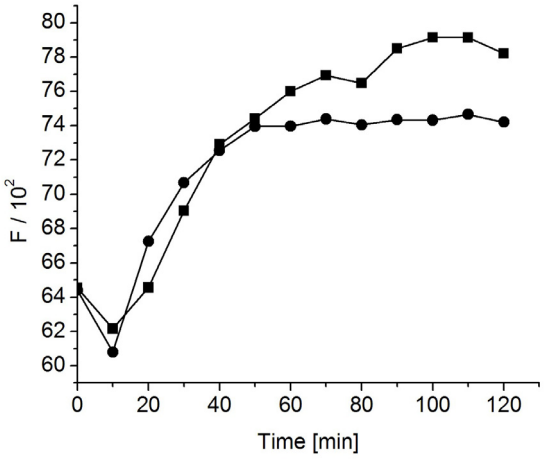


Figure 35: Only a 0.3- or 0.2-fold increase in fluorescence is observed when 70 nM pre-bantam beacon **31** (■) or 40 nM FAM pre-mir-21 beacon **33** (●) is incubated with 1 U Dicer (Invitrogen) under optimized buffer conditions.

Another method was chosen to introduce the DABCYL group to the 3′-terminus *via* ligation of a 3′,5′-bisphosphate using T4 RNA ligase.[147, 148, 149] A DABCYL-modified cytidine 3′-5′-bisphosphate **39** (DABCYL pCp) was selected, the synthesis of which can be seen in Figure 36.

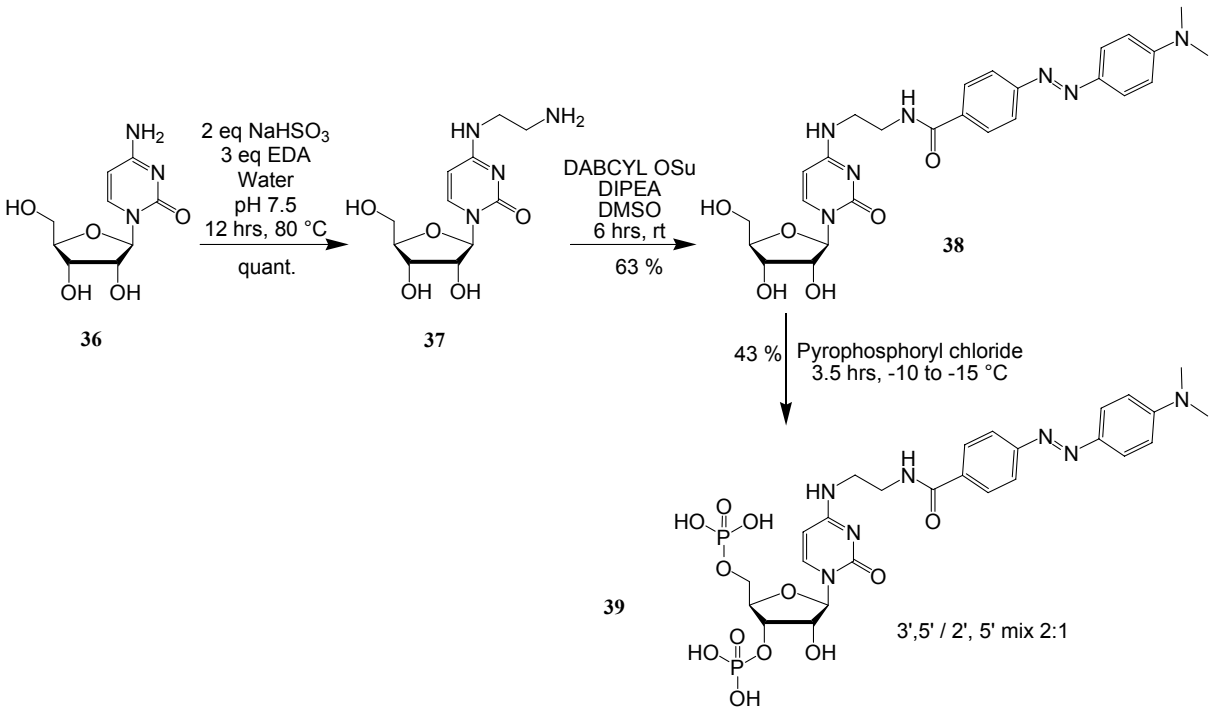


Figure 36: Three step synthesis of DABCYL cytidine 3′(2′),5′-bisphosphate **39**. DABCYL OSu ≡ DABCYL N-hydroxysuccinimide ester.

The synthesis of **39** is straightforward and simple, providing good yields. The difficulties lie in the purification steps. Starting from commercially available cytidine **36** the transamination product **37** is easily synthesized with only 2 eq sodium hydrogensulfite and 3 eq ethylenediamine at 80 °C. This is significantly less than in the literature (12.5 M excess $NaHSO_3$, 20 M excess diamine) and simplifies the purification.[173] The longer diamines 1,4-diaminobutane and 1,10-diaminodecane were also used successfully in the transamination reaction (data not shown). It is known that the longer diaminoalkanes react more slowly,[174] however running the reaction at 80 °C increases yield and lowers both reaction time and side products (uracil, uridine) as determined by TLC. Higher temperatures were not attempted. The water soluble product was desalted by gel purification (Bio-Gel polyacrylamide gel, Bio-Rad). Since remnants of ethylene diamine could reduce yield in the subsequent reaction, a second FC purification step was performed using normal silica gel in MeOH. Solubilized silica gel was removed by precipitation from water. Coupling of the DABCYL group was done with the *N*-hydroxysuccinimide ester thereof in the presence of base and was complete within one hour as determined by TLC. Normal FC purification gave the product **38** in acceptable yields. The final phophorylation step in neat pyrophosphoryl chloride at -15 °C is a known procedure giving a 3′(2′), 5′-bisphosphate mixture (2:1). The 2′,5′-bisphosphate side product is neither substrate nor inhibitor of T4 RNA ligase.[175]

Dabcyl pCp **39** was successfully coupled to the 5′-FAM let-7 transcript to give **32**. The low 10% yield in this case is most likely a combination of RNAse contamination from the pCp derivative as well as loss of the 5′-FAM group during the final 3′-desphosphorylation step using calf intestinal phosphatase (CIP). Further ligation reactions yielded the desired product quantitatively in the presence of RNAse inhibitor. The FAM group is attached *via* an aminohexyl phosphate linker, which was observed to also be a substrate for CIP (yellowish color in phenol phase). This was also observed during dephosphorylation of the synthetic FAM-only pre-let-7 beacon **11**. This can easily be avoided by dephosphorylation in the presence of polynucleotide kinase (PNK) in the absence of ATP.[176] The 3′-phosphatase activity of PNK is much less than that of CIP, but can nonetheless be successfully applied. Beacons FAM pre-mir-21 **34** and TMR pre-mir-21 **35** were successfully synthesized using this method (see below).

The final pre-let-7 beacon **32** was processed very well by Dicer, resulting in a 6.4-fold fluorescence increase over 4 hours (Figure 37). This is even greater than the typical 3-fold fluorescence increase seen with the synthetic let-7 beacon **10** at the same concentration. This

could be due to the structure of the synthetic beacon, which contains a 1-nt 3′-overhang of a deoxycytidine DABCYL derivative and might not be accepted as well as the more 'natural' structure with a 2′-hydroxyl group. Beacon **32** also contains a shorter linker between the base and the DABCYL group (C2 compared to C12), which might allow better Dicer processing of this beacon. This might also benefit fluorescence quenching in the hairpin structure.

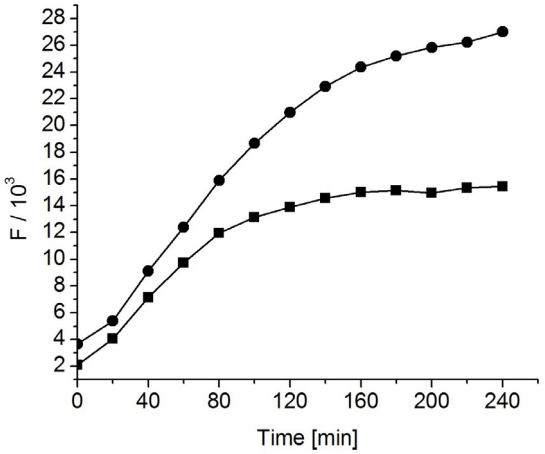


Figure 37: Fluorescence increase of FAM pre-let-7 beacon **32** in the presence of 0.1 U Dicer in optimized buffer was 6.4-fold (± 0.4) at 20 nM (■) and 6.4-fold (± 0.2) fold at 40 nM (●).

A criticism might be the inhomogeneous 3′-terminus obtained from the *in vitro* transcription reaction, which does not represent the natural pre-miRNA structure containing a 2-nt 3′-overhang. However, it is known that Dicer processes overhangs of 1-3 bases producing 21-23 mers, which is in agreement with data thus presented (see previous sections).[46] Since any potential binders of pre-miRNA would most likely show inhibitory effects through binding to internal sites, an inhomogeneous 3′-terminus should not be a problem.

The FAM- and TMR-labeled pre-mir-21 beacons **34** and **35** were also synthesized. A DNA template containing 2′-*O*-methyl modifications on the last two bases was used to help reduce overrun transcription.[177] Both beacons were made using *in vitro* transcription with either a 5′-FAM- or 5′-TMR-ApG transcription starter. The lipophilicity of TMR allowed somewhat better HPLC purification of the TMR-labeled transcript. After ligation of pCp-DABCYL **39**, removal of the 3′-phosphate was carried out using PNK in the presence of RNAse inhibitor instead of CIP, resulting in quantitative recovery of product while ensuring complete removal of the phosphate group as determined by MALDI-TOF. Processing by rhDicer of the FAM pre-mir-21 beacon **34** with or without a 3′-phosphate resulted in a 1.50 ± 0.04 or 1.67 ± 0.02

fold fluorescence increase over 4 hours, respectively (data not shown). Thus, the 3′-phosphate does not seem to significantly affect Dicer's ability to process the pre-miRNA.

Incubation of beacons **34** and **35** together in the duplex format also resulted in no significant difference in processing by rhDicer (1.75 ± 0.12 vs 0.67 ± 0.10 fold increase over 4 hours) as compared with processing of each beacon alone (1.70 ± 0.07 vs 0.70 ± 0.07 fold increase over 4 hours). However, both of these beacons possess a 3′-overhang of at least 3-nt, which results in lower processing of the beacons by rhDicer (Figure 38). Thus, the difference in fluorophore (FAM or TMR) does not appear to have an effect on Dicer cleavage of the beacon.

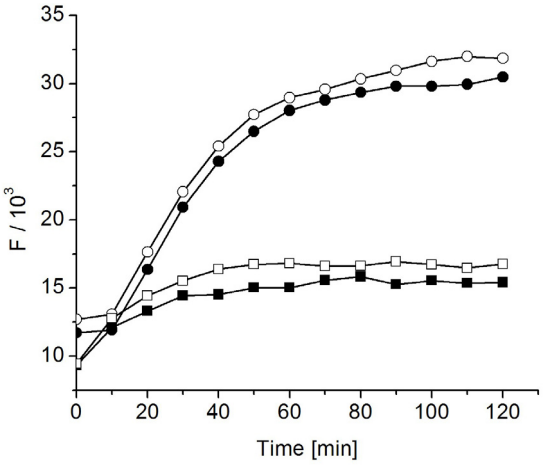


Figure 38: Duplex assay compared to individual measurements: FAM-mir-21 **34** alone (○) or in duplex format (●); TMR-mir-21 **35** alone (□) or in duplex format (■). Averages of triplicate measurements using optimized conditions included 40 nM beacon, 0.1% Triton X-100, and 1 U recombinant Dicer.

3.6 Cellular miRNA Maturation Assay

With the assay established and various pre-miRNA binders tested *in vitro* the assay was then tested in living cells. Working together with the group of Prof. Andreas Herrmann at the Humboldt-Universität zu Berlin the assay was first tested in HEK 293 cells using either the FAM pre-mir-142 beacon **12** or the Cy3 mir-19b-2 beacon **14**. After 24 hours a definite fluorescence increase could be seen with both beacons (Figure 39). The Cy3 fluorescence is much stronger than that of FAM. The fluorescence also tapers off after 24 hours with FAM and remains above the detectable threshold with Cy3 (Figure 40).

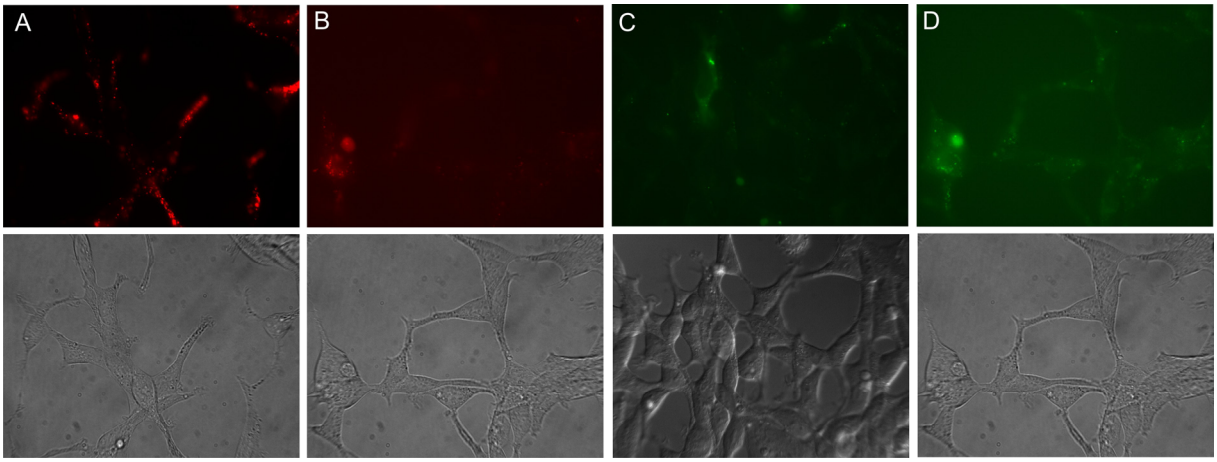


Figure 39: Fluorescence (false color) observed after 24 hours in HEK 293 cells transfected with either Cy3 mir-19b-2 beacon **14** (A), FAM mir-142 beacon **12** (C), or control siRNA (B/D). Fluorescence in controls was simply measured at the respective emission wavelength of either Cy3 or FAM. Fluorescence (top) and contrast (bottom) pictures are given in each case. Pictures C are from a separate experiment.

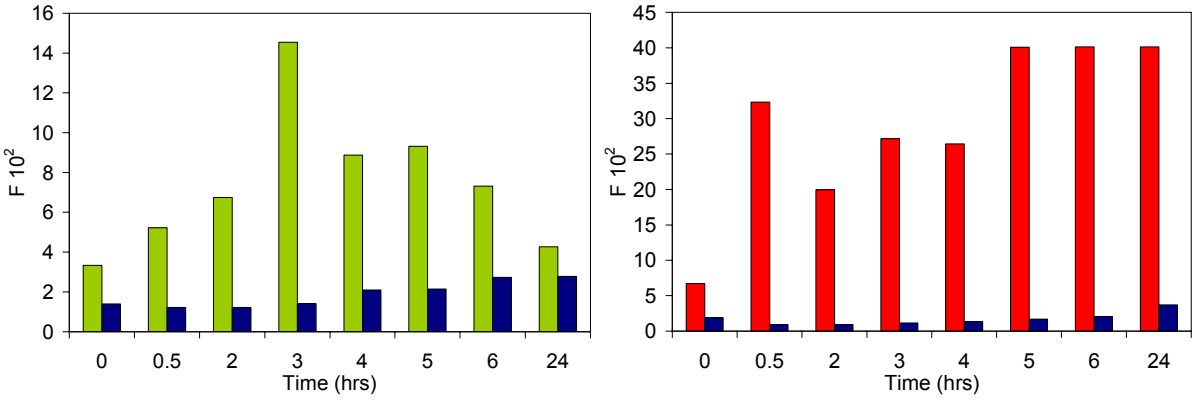


Figure 40: Fluorescence over time in cells transfected with FAM mir-142 **12** (left, green) or Cy3 mir-19b-2 **14** (right, red). Blue columns represent self-fluorescence at respective wavelength (520 nm/FAM, 563/Cy3) of cells transfected with control siRNA. Data are the average of total epifluorescence of two separate images at each time point.

Dicer knockdown cells were then used to show that the fluorescence increase is Dicer-dependent. Cells were treated with Dicer siRNAs on days 1 and 3. Cells were then transfected with the Cy3 beacon **14** on day 4 as previously described and the fluorescence observed every 10 min. with a confocal microscope. The difference in fluorescence between knockdown and untreated cells is quite apparent (Figure 41).

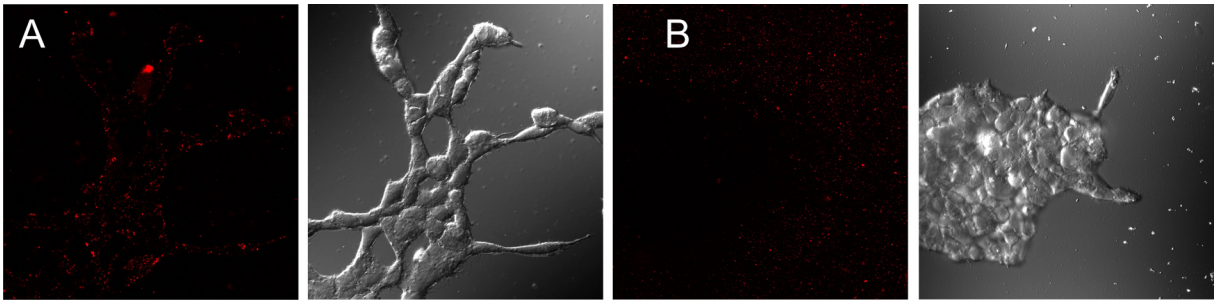


Figure 41: Fluorescence (false color) after 2 hours in Dicer knockdown cells (B) and untreated cells (A) transfected with Cy3 pre-mir-19b-2 beacon **14** as measured with a confocal microscope.

A specific region of cells was observed over the course of 2 hours and the integrated intensity over the entire region analyzed. Untreated cells showed a doubling of the fluorescence over the course of 2 hours while knockdown cells only increased by 0.3-fold (Figure 42).

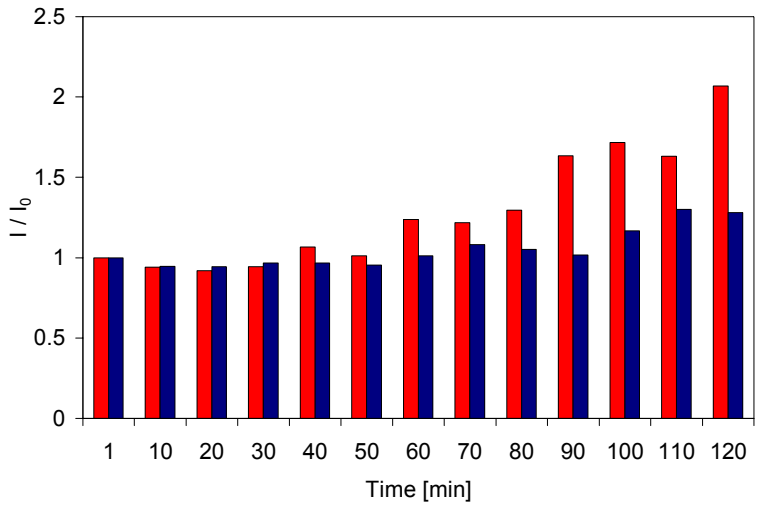


Figure 42: Fluorescence (I/I_0) of Cy3 pre-mir-19b-2 beacon **14** observed in untreated cells (red) and cells treated with siRNA against Dicer mRNA (blue). Data are the integrated fluorescence intensities of a single cell region over time as measured using a confocal microscope. I = fluorescence intensity at given time point. I_0 = initial fluorescence intensity.

In order to verify the knockdown efficiency of Dicer a Western blot of harvested knockdown cells was done in a separate experiment. Cells were treated as for the confocal experiment and a Western blot carried out with the cell lysates. Unfortunately, no signal at the appropriate mass of Dicer (220 kDa) could be seen in the blot even with extensive exposure of the film.

Neither controls (untreated cells) nor knockdown cells provided observable levels of protein (data not shown). Either the level of Dicer protein was below the detection limit or Dicer was degraded to the point that detection was impossible.

Validation of the confocal fluorescence measurements of the knockdown cells can also be done using RT-PCR of the cell lysates. As control a typical 'housekeeping' gene is also run to make sure the PCR was successful. This will be examined more closely in the future.

3.7 Modified RNA Building Blocks for miRNA-Dicer Cross-links

Developing specific small molecule binders of pre-miRNA would be aided significantly by a thorough understanding of the cleavage mechanism of Dicer as well as the interaction of the enzyme in general with the RNA hairpin structure. Recent insights into the structure of Dicer have allowed a better understanding of how Dicer interacts with dsRNA.[41, 42, 43, 47] Another method to study the interactions of Dicer enzyme with the pre-miRNA would be through cross-linking studies. RNA can be cross-linked with nearby regions in the protein by covalent means or non-covalently. RNA modified at specific positions with reactive groups can be incubated with the desired protein to allow a covalent linking with nearby regions in the protein.[178, 179] After partial degradation of the protein with protease the fragments bound to the RNA can be analyzed (e.g. with mass spectrometry or peptide sequencing) to determine which regions interact with the RNA.[180, 181]

The first step towards such studies is the synthesis of modified RNA phosphoramidites needed to synthesize the reactive RNA probes. Modification at the 2′-position was chosen as this should come into close contact to amino acid side chains on the enzyme during Dicer processing without necessarily being directly involved in the cleavage mechanism itself. A simple primary amino terminus can be coupled in the presence of an activator (e.g. 1-ethyl-3-dimethyl-aminopropylcarbodiimide or dicyclohexylcarbodiimide) with an aspartate or glutamate amino acid side chain in the protein.[182, 183] A thiol group can react with cysteines near the active center.[184, 185, 186, 187, 188] Both reactions rely on close proximity of the amino acid residue to the modified nucleotide. Alternatively, both groups can be modified with more reactive groups in solution such as N-hydroxysuccinimide (NHS) esters or maleimide reagents, respectively. A wide variety of cross-linking reagents are commercially available.[189]

Formation of a 2′-methylaminouridine derivative was chosen because of the simple synthetic route.[190] Additionally, the methyl group should prevent any possible side reactivity at the 3′-phosphate.[190] The amino terminus can then be easily extended with the desired modifications using acid chlorides before final conversion to the phosphoramidite.

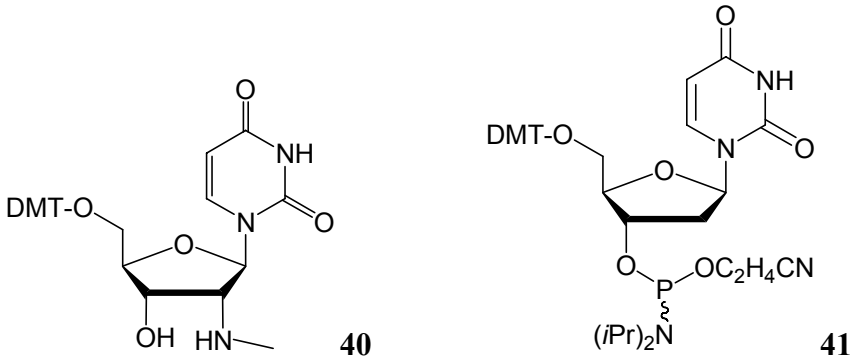
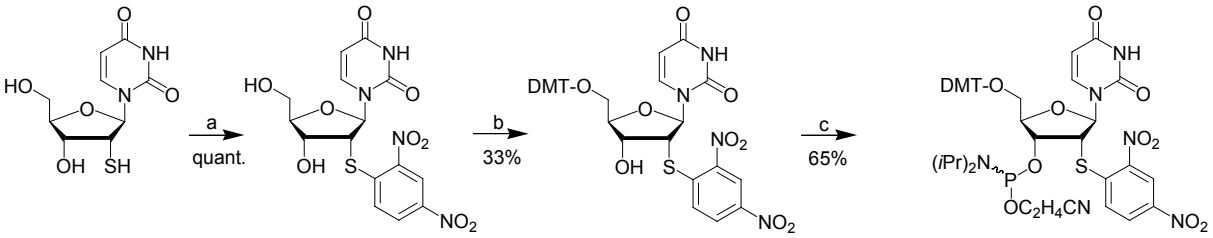


Figure 43: 2′-methylamino modification of uridine (left) and 2′-deoxyuridine phosphoramidite (right).

A simple 2′-thiouridine phosphoramidite derivative was also seen as a possible route for cross-linking studies.[191] The free 2′-thiol can either be modified in advanced with the desired chemistry or modified later directly on the RNA using a maleimide coupling reagent containing the desired modification. The 2,4-dinitrobenzene group was chosen as protecting group for the thiol. The synthesis is relatively short and simple, starting from the known 2′-deoxy-2′-thiouridine **42** (Figure 44).



42 **43** **44** **45**

Figure 44: Synthetic route towards **45**: a) Fluoro-2,4-dinitrobenzene, aqueous buffer; b) DMT-Cl, pyridine; c) Phosphitylating reagent **48**, DCI/ACN.

Stability of the intermediate compound **43** to oxidative conditions was tested using a standard 0.1 M iodine oxidation reagent used in automated nucleic acid synthesis. Additionally, stability towards acidic conditions was also tested using glacial acetic acid. In both cases no

degradation products could be observed by TLC after several hours treatment in either reagent. The 2′-thiouridine phosphoramidite **45** was then used in the solid-phase synthesis of the short 5 mer starting from DMT-G^{Ac}-cpg (Figure 45). For this synthesis the simple 2′-deoxyuridine phosphoramidite **41** was also used (Figure 44).

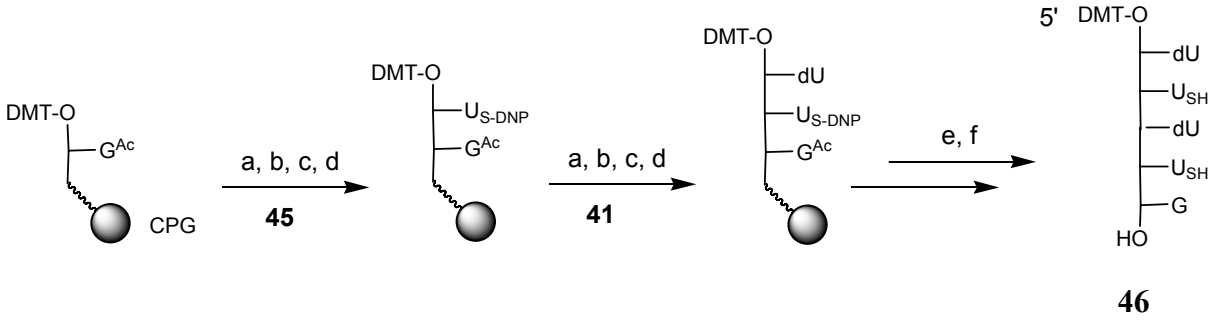


Figure 45: Synthetic steps of **46**: a) TCA/DCM; b) Coupling in 0.25 M DCI; c) Cap A/B; d) I_2 oxidation; e) Multiple rounds of steps a-d; f) Cleavage and deprotection with MA solution. DNP ≡ dinitrophenyl.

Methylamine cleavage reagent was used to cleave the RNA from cpg as well as remove the acetyl protecting group on G. MALDI-TOF analysis of the crude product showed that the dinitrobenzene protecting group was also removed, presumably during the basic deprotection (Figure 45). This however proved to be unproblematic as no degradation fragments owing to nucleophilic attack of the 2′-thiol at the 3′-phosphate could be seen during MALDI-TOF analysis. This is supported by evidence from various studies on 2′-thiol derivatized systems.[192, 193] Additionally, no product dimerization was observed.

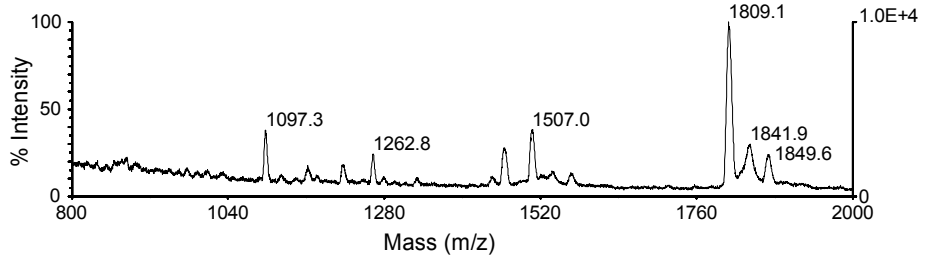


Figure 46: Crude **46** after cleavage from cpg and deprotection in MA solution.

A 2′-thiouridine analog was found to be more stable than its 2′-hydroxyl homolog at various pH values. This is due to its overall lower reactivity towards the 3′-phosphate group, the thiolate preferring equatorial versus apical attack at the 3′-phosphate. The preferred

degradation is attack of the 2′-thiolate at the 1′-carbon leading to concomitant release of the uracil base under highly basic conditions (pH > 14) and subsequent strand cleavage.[192]

A test coupling was then carried out using *N*-(1-pyrenyl)maleimide. To 4 µL crude 5 mer RNA **46** in 1 mM ammonium citrate, pH 7.0 (~60 µM) was added 1µL 20x TE buffer and either 1 µL 10 mM DTT or 1µL 100 mM TCEP and incubated at rt for 1 hour before adding 18 µL DMSO followed by 1 µL 100 mM *N*-(1-pyrenyl)maleimide in DMSO. After mixing, the reactions were incubated at 60 °C for 2 hours. After salt exchange *via* drop dialysis (Millipore filter discs, #VSWP01300) of a small volume of 2-5 µL against 1 mM ammonium citrate buffer the reactions were analyzed by MALDI-TOF. The desired product containing two coupled pyrene groups could be seen (calc. $[M]^{-1}$ 2402, found 2407) in addition to only one coupled pyrene (calc. $[M]^{-1}$ 2105, found 2108). Remaining unmodified 5 mer **46** could be seen as well as some degradation products (Figure 47). More degradation was observed in the reaction containing DTT although the overall peaks were stronger in intensity compared to the reaction performed with TCEP as reductant. This could also simply be due to a less efficient desalting prior to MALDI-TOF analysis, this being the greatest difficulty in detecting such a small nucleic acid fragment. When no reductant was used only minimum pyrenyl-coupled product could be detected, which is interesting, since the educt 5 mer showed at no time a tendency towards oxidation.

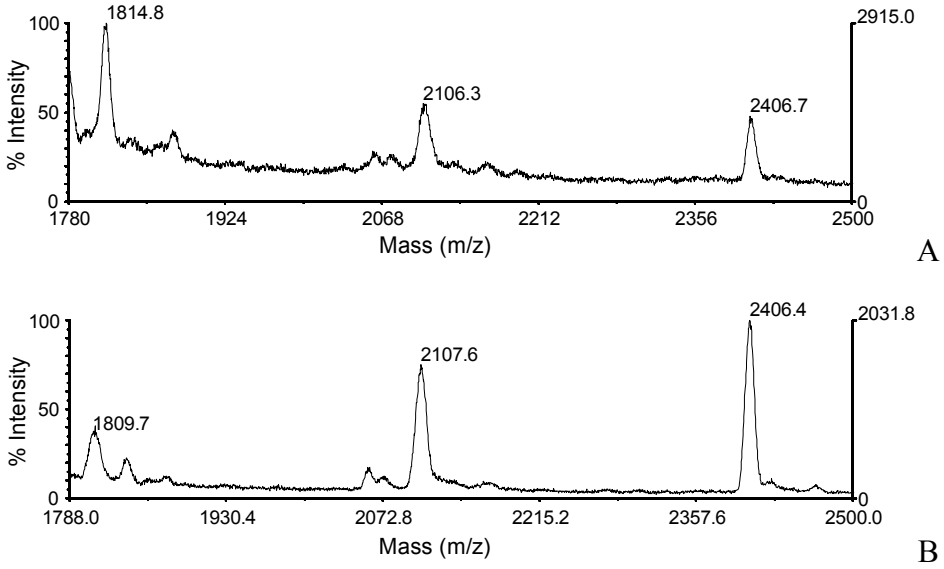


Figure 47: Coupling of pyrenyl maleimide to **46** in the presence of TCEP (A) or DTT (B).

The low yield of this reaction is expected due to the poor water solubility of the pyrene maleimide reagent and its steric bulkiness. Nonetheless, the successful coupling to the 2′-

thiols shows that the 2′-thiol indeed is an appropriate modification for attachment of maleimide reactive groups. Also, the 2,4-dinitrobenzene group can be successfully used as protecting group of the 2′-thiouridine as opposed to the trityl group.[191]

4 Summary and Outlook

The first homogenous assay of miRNA maturation has been presented here. A fluorescence-based probe of miRNA maturation was successfully synthesized and was shown to be processed efficiently by the key enzyme in the RNAi / miRNA pathway Dicer. The assay was used successfully to demonstrate that various substances (Kanamycin A, peptides, small molecules) are capable of inhibiting Dicer-mediated miRNA maturation *in vitro*.

The assay in its original form, however, does not allow a prediction of whether the inhibition of maturation is selective for a single miRNA species. In fact, using this assay it is even impossible to differ between a selective miRNA inhibitor and an inhibitor of the Dicer nuclease itself, which would lead to a reduced maturation of all cellular miRNAs simultaneously. A duplex assay was thus developed, which was used to show some selectivity in inhibiting maturation of a specific miRNA. Evidence for the supposed mode of inhibiting miRNA maturation is also supported by SPR binding experiments. Furthermore, the assay was tested successfully in a cell line, so that potential inhibitors can be examined under more natural conditions. Finally, the beacons were synthesized both by chemical means as well as using *in vitro* transcription together with enzymatic ligation, providing a cost-efficient and viable method for the production of a collection of different miRNA maturation probes.

The next step is to test more substances using the assay in order to further validate its usefulness as well as gain more insight into what kind of substances provide the most potent inhibition of miRNA maturation. The results from SPR studies showed a strong correlation between RNA binding (K_D) and inhibition (IC_{50}), however use of the duplex assay format should be ideal for achieving specific blockage of miRNA function. Further methods might be used to correlate RNA binding with maturation inhibition including gel shift assays and isothermal titration calorimetry.

Although it is known that binding at or near the Dicer cleavage sites inhibits cleavage,[134] further work is needed to determine what other RNA structural elements might be sensitive to ligand binding in respect to Dicer cleavage. RNA footprinting with selected binders might aid in this search. The binder is incubated with the RNA and subsequently digested by different RNAses. The cleavage of the RNA is reduced at those epitopes tightly bound by the ligands. The respective RNA regions can be revealed by analysis (PAGE, MALDI-TOF) of the cleavage fragments.

Once specific binders are found their inhibitory effects can also be tested in cells using this assay. Additionally, complementary methods of studying the interaction of pre-miRNA binders in the cell are needed. Just such a method might be reporter gene assays. An example of a fluorescence-based reporter gene assay developed by the Tuschl group was originally developed to monitor the function of mir-21 in HeLa cells, although testing of antisense nucleic acids (e.g. antagomirs) for their inhibitory effect is also feasible with the method.[194]. Similar to the widely used luciferase reporter gene assays they constructed plasmids containing the desired miRNA sequence in the 3′-UTR of enhanced green fluorescent protein (EGFP) thus leading to a miRNA-mediated silencing of the reporter gene. When transfected along with a 2′- *O*-methylated antisense molecule against mir-21, fluorescence was seen. Fluorescence was not observed when either the sense strand or the unmodified antisense strand was used. It might be possible to also test for small molecule inhibitors of miRNA maturation using such an assay. A drawback with such a system, however, is that no information on the mode of action is provided even if fluorescence is observed.

It would be interesting to see the effect of reversing the positions of fluorophore and quencher (i.e. 5′-quencher and 3′-fluorophore). Additionally, the assay might be further enhanced through the use of FRET. By using the proper fluorophore combination (e.g. FAM / TMR) a positive signal would be assured both with the intact beacon as well as after cleavage by Dicer. Positive fluorescence might also be achieved through excimer fluorescence as is known with pyrenes.[195, 196] The pre-miRNA structure might allow just such an interaction.

Designing potent inhibitors of pre-miRNA maturation could be aided through a better understanding of the interaction between Dicer and the pre-miRNA. Using the modified nucleotide chemistry described here Dicer might be cross-linked to the pre-miRNA at various positions. Understanding which amino acid residues interact with the RNA might aid in the development of more potent miRNA maturation inhibitors.

Testing of large libraries is needed to find selective inhibitors of miRNA maturation.[197] The ever-increasing amount of research into RNA interactions with its environment should aid this search. With the discovery of miRNAs the ‘RNA world’ has taken on entirely new dimensions.[198] The importance of RNA in biological processes has been perhaps underestimated for many years, but its large role in the cell has been at the latest now recognized.[199]

5 Experimental

5.1 General Materials and Methods

Chemicals

All reagents used were of the highest quality available and certified pyrogen / DNAse / RNAse free when possible. Unless otherwise noted reagents were from Acros (Geel, Belgium), Aldrich/Fluka/Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or AppliChem (Darmstadt, Germany). Acetonitrile for HPLC was obtained from LGC PromoChem (Wesel, Germany). Water was ultra purified using an Astacus Life Science water purification system from membraPure (Bodenheim, Germany). This water was tested and determined to be free of RNAse contamination as shown by denaturing PAGE analysis of a sample RNA transcript (data not shown). All solvents for RNA synthesis were supplied by Applied Biosystems (USA) and used as purchased. Solvents for other chemical syntheses were dried according to standard methods when needed.[200] 2′-TOM-protected RNA base phosphoramidites, DABCYL-dU phosphoramidite ('DABCYL dT'), (6)FAM phosphoramidite, 5′-phosphorylating reagent (CRI, #10-1900-90), universal support II polystyrene (USIIPS), and RNA cpg were purchased from Glen Research (Sterling, VA USA). (6)TMR-C_{12} phosphoramidite was obtained from Biosearch Technologies (Novato, CA USA). Ribonucleosides were purchased from ChemGenes Corp. (Wilmington, MA USA). Benzylmercaptotetrazole was obtained from EMP Biotech (Buch, Germany). Dicer was purchased from Stratagene (La Jolla, CA, USA), Genlantis (San Diego, CA, USA), Ambion (Austin, TX, USA) and Invitrogen (Carlsbad, CA, USA). Polystyrene microtiter plates (black, non-coated) were supplied by Greiner (Frickenhausen, Germany).

General procedures for working with RNA

Any glassware, metal or other materials that were used for RNA work were either heat treated (250 °C overnight) or treated with sodium hypochlorite solution (3% household bleach) or 0.25 M NaOH solution and then rinsed thoroughly with ultra purified water before use. All surfaces were regularly treated with 3% household bleach solution and then rinsed with ultra purified water. Disposable gloves were always used and rinsed first with a 1% household bleach solution (diluted from 3% commercial bleach using distilled water) and patted dry using commercially available bleached absorbent paper towels. All buffers for RNA work

were sterile filtered through 0.22 µM filters. All materials that could be purchased certified Pyrogen / DNAse / RNAse free were done so.

Chromatography

FC was performed using silica gel 60 (63-200 µM) and TLC using aluminum plates coated with silica gel 60 (F_{254}) from Merck (Darmstadt, Germany). In addition to UV light (254 nm) various staining reagents were used to detect the desired compounds on TLC including:

Seebach's reagent: 25 g Phosphomolybdic acid, 10 g cerium (IV) sulfate tetrahydrate, 60 mL H_2SO4, 905 mL H_2O

Ninhydrin: 1 g ninhydrin in butanol with 3% acetic acid

Sugar reagent: 5% (v/v) H_2SO_4 in EtOH

HPLC

Reverse phase HPLC was performed on a Gilson 321 HPLC (Middleton, WI USA) using a Polaris column from Varian, Inc. (5µm, 200Å, 250 mm x 4.6 mm) with UV detection at 260 nm and fluorescence excitation / emission detection (495 / 520 FAM, 556 / 580 TMR, 546 / 563 Cy3). Columns were heated at 55 °C. Acetonitrile gradients (eluent A) were used in 0.1 M TEAA (eluent B) with a flow rate of 1 mL / min. Extinction coefficients for the RNA were calculated using the nearest neighbor method.[201] The extinction coefficients for beacons were calculated as the sum of the unmodified RNA together with the extinction coefficients for 5′-FAM (ε_{260} = 21,000), dC-DABCYL / dU-DABCYL / C-DABCYL (ε_{260} = 29,100), TMR (ε_{260} = 32,000) and Cy3 (ε_{260} = 5,000). Hypochromicity effects due to hairpin formation were not taken into account. All yields for RNA strands were calculated by integration of the HPLC chromatograms.

NMR

All measurements were carried out using a Bruker 300 MHz (^{1}H at 300 MHz, ^{13}C at 75 MHz, and ^{31}P at 121.4 MHz) spectrometer at room temperature. ^{31}P spectra were calibrated with H_3PO_4 as external reference. Chemicals shifts (δ) are given in ppm and all spectra were calibrated using the residual solvent signals.[202] Coupling constants *J* are given in Hertz

(Hz) using the abbreviations: s = singulet, bs = broad singulet, d = doublet, t = triplet, q = quadruplet, dd = doublet of doublets, m = multiplet.

Mass spectrometry

MALDI-TOF measurements were performed on a Voyager-DE Pro Biospectrometry Workstation from PerSeptive Biosystems (Foster City, CA USA) containing a nitrogen UV laser (λ = 337 nm) for excitation. Measurements were done in linear and negative or positive ion mode. The settings for RNA consisted of an accelerating voltage of 25 kV, a grid voltage at 93.5%, a guide wire voltage at 0.15% and a delay time of 400 ns. For small molecule measurements an accelerating voltage of 22 kV, a grid voltage at 95%, a guide wire voltage at 0.15% and a delay time of 400 - 600 ns were applied. A matrix THAP-citrate in a ratio of 2:1 v/v was made from 0.3 M THAP (2,4,6 trihydroxyacetophenone) in EtOH and 0.1 M ammonium citrate (pH 6.4) in water and used for all measurements. Calculated masses are average masses, but m/z peaks found are those of the unresolved pseudomolecular ions $[M-H]^-$ or $[M+H]^+$.

Low resolution mass spectrometric measurements were done using electrospray ionization (ESI) with an ion trap detector on a Thermo mass spectrometer. For high resolution measurements a Thermo LCQ FT-ICR (Fourier transform ion cyclotron resonance) mass spectrometer with electrospray ionization was used.

Spectrophotometric measurements

UV-Vis concentration measurements were done on a NanoDrop ND-1000 spectrophotometer (Wilmington, DE USA).

Fluorescence measurements were done either on a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA USA) or a BMG Labtech Fluorostar Optima plate reader (Offenburg, Germany). Excitation / emission filters used for the BMG plate reader included 485 / 520 nm (FAM) and 540 / 575 nm (Cy3, TMR) all with slit widths of 10 nm.

Calculation of inhibition rates / IC_{50} values for RNA binders: To calculate the percent inhibition of a given substance at a specified concentration the average of at least two measurements was used. The Dicer-mediated (D) fluorescence increase during the specified

time range either in the presence ($F_{D,TS}$) or absence (F_D) of a test substance (TS) was calculated by linear regression according to the following equation:

$$Inhibition(\%) = \left(1 - \frac{\partial F_{D,TS}}{\partial F_D}\right) x100$$

IC_{50} values were calculated from the average of 3 measurements at 3 or 4 concentrations. The percent inhibition over the specified time range at each given concentration of test substance was calculated as described above and the IC_{50} value determined using least squares analysis from these data.

5.2 Chemical Synthesis

5.2.1 Small Molecule Synthesis

Bis-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (47): Phosphorylating reagent **47** was prepared as described with slight modifications.[203] To a stirred, 0 °C cooled solution of hydroxypropionitrile (8.1 g, 114 mmol, 7.8 mL) and DIPEA (21.2 g, 170 mmol, 27.1 mL) in dry THF (40 mL) was added diisopropylaminophospohorous dichloride (11.5 g, 57 mmol) dropwise over 15 min. The solution was stirred another hour at rt. The amine·HCl salt was filtered off under argon and the THF removed from the filtrate under vacuum. Kugelrohr distillation was attempted, but the product was finally purified by FC on silica gel (cyclohexane / ethyl acetate / TEA 80:15:5) giving a slightly yellow oil (1.45 g, 5.35 mmol, 9%). ^{1}H NMR[204] (CD_3CN): δ = 1.18 (2d, *J* = 6.8, 4 -CH_3), 2.66 (t, *J* = 6.0, 2 -CH_2CN), 3.63 (m, 2 -C*H*CH_3), 3.81 (m, 2 -OC*H*$_2$$CH_2CN$); ^{13}C NMR ($CD_3CN$): δ = 20.9 (d, 2 -*C*$H_2CN$), 24.8 (d, *J* = 7.4, 4 -CH_3), 43.8 (d, *J* = 12, 2 -*C*HCH_3), 59.4 (d, *J* = 19, 2 -O*C*H_2CH_2CN), 119.4 (s, -CN); ^{31}P NMR[203] (CD_3CN): δ = 148.5.

2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite (48): Phosphitylating reagent **48** was prepared as described with the following modifications.[205] Phosphorus trichloride (27.5g, 0.2 moles, 17.5 mL) was added to a cooled (0 °C), stirred mixture of ACN (250 mL) and diisopropylamine (121 g, 1.2 mol, 168.7 mL) over 30 min. After stirring for another 30 min. at rt, 250 mL cyclohexane was added to the thick, yellowish mixture. Hydroxypropionitrile (14 g, 0.2 mol, 13.5 mL) was added over 30 min. while swirling. The reaction mixture became somewhat less viscous and was stirred for another 1 hour before

filtering under argon to remove the amine salts. The upper cyclohexane layer from the filtrate was allowed to separate (30 min.) and was then siphoned off using a canula and the solvent removed under vacuum (Fraction 1). Another 200 mL cyclohexane were added to the ACN phase and stirred vigorously for 2 hours. After separation, the cyclohexane phase was siphoned off as above and the solvent removed (Fraction 2). Both fractions 1 and 2 were distilled separately by Kugelrohr distillation. Fraction 1 gave a slightly yellowish, thick oil (9.3 g, 0.034 mol). Fraction 2 gave a nearly colorless, thick oil (27 g, 0.1 mol). Total yield: 45%. ^{1}H NMR[206] (CD_3CN): δ = 1.17 (2d, *J* = 6.9, 8 -CH_3), 2.63 (2t, *J* = 6.0, -CH_2CN), 3.56 (m, 4 -C*H*CH_3), 3.72 (2t, *J* = 6.0, -OC*H*$_2$$CH_2CN$); ^{13}C NMR ($CD_3CN$): δ = 21.1 (d, -*C*$H_2CN$), 24.1 (d, *J* = 6, 4 -$CH_3$), 24.8 (d, *J* = 8, 4 -$CH_3$), 45.2 (d, *J* = 12, 4 -*C*HCH_3), 60.3 (d, *J* = 24, -O*C*H_2CH_2CN), 120 (s, -CN); ^{31}P NMR[207] (CD_3CN): 123.9.

2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine (49): Compound **49** was prepared similarly as described.[208, 209] 2′-Deoxyuridine (200 mg, 0.877 mmol) was dissolved in dry pyridine (2 mL) containing molecular sieves. 4,4′-Dimethoxytrityl chloride (327 mg, 0.965 mmol) dissolved in 2 mL pyridine was added slowly over 3 hours. After 4 hours the reaction was quenched with methanol. The mixture was diluted with DCM and the organic phase washed twice with 0.1 M ammonium acetate, once with brine, dried over $MgSO_4$, and the solvent removed under vacuum. The crude product was purified by FC (DCM flush, then DCM / MeOH 20:1). Removal of the solvent under vacuum gave colorless foam (335 mg, 72%). R_f = 0.31 (DCM / MeOH 95:5 with 1% TEA); ^{1}H NMR (CD_3CN): δ = 2.24 (m, H2′, H2″), 3.29 (m, H5′), 3.43 (d, *J* = 4.3, 3′-OH), 3.76 (s, 6 -OCH_3), 3.93 (m, H4′), 4.44 (m, H3′), 5.36 (d, *J* = 8.1, H5), 6.16 (t, *J* = 6.3, H1′), 6.87 (m, 4 Ar-H), 7.2-7.27 (m, Ar-H), 7.27-7.35 (m, 6 Ar-H), 7.35-7.45 (m, 2 Ar-H), 7.63 (d, *J* = 7.6, H6), 9.30 (s, NH); ^{13}C NMR (CD_3CN): δ = 41.0 (C2′), 55.9 (-OCH_3), 64.1 (C5′), 71.5 (C3′), 85.7 (C1′), 86.8 (C4′), 87.4 (5′-OCDMT), 102.4 (CH5), 114.1 (2s, Ar-CH), 127.9 (Ar-CH), 128.9 (Ar-CH), 129.0 (Ar-CH), 131.0 (Ar-CH), 136.5 (Ar-C), 136.7 (Ar-C), 141.4 (CH6), 145.9 (Ar-C), 151.4 (C2), 159.7 (Ar-C), 164.2 (C4).

2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite) (41): 49 (335 mg, 0.631 mmol) was dissolved in DCI activator (6mL, 0.25 M in ACN). Compound **48** (381 mg, 1.26 mmol, 401 µL) was added under stirring at rt. After 1 hour another 200 µL **48** were added and stirred for another 30 min. The reaction mix was taken up in DCM and extracted 3x with 5% $NaHCO_3$, once with brine

and then dried over $MgSO_4$ and the solvent reduced under vacuum to several mL. This volume was dropped slowly under vigorous stirring to 250 mL pentane forming a colorless precipitate. After storage at -20 °C overnight the organic phase was decanted and the sticky precipitate dried under vacuum giving a colorless foam (450mg, quant.). R_f = 0.43 (DCM/MeOH 95:5 with 1% TEA); ^{31}P NMR (CD_3CN): δ = 148.3, 148.4. LRMS calc. for $C_{39}H_{48}N_4O_8P$ $[M+H]^+$ 731.3, found 730.8.

2,2′-*O*-Anhydro(β-D-arabinofuranosyl)uracil (50): Compound **50** was prepared as described in 72% yield.[210] R_f = 0.22 (DCM/MeOH 85:15); ^{1}H NMR (DMSO-d_6): δ = 3.23 (m, 2-H5′), 4.07 (m, H3′), 4.38 (s, H4′), 4.99 (t, *J* = 4.9, 5′-OH), 5.20 (d, *J* = 5.8, H2′), 5.84 (d, *J* = 7.4, H5), 5.90 (m, 3'-OH), 6.30 (d, *J* = 5.7, H1'), 7.84 (d, *J* = 7.5, H6). ^{13}C NMR[211] (DMSO-d_6): δ = 60.9 (C5′), 74.7 (C3′), 88.8 (C4′), 89.2 (C2′), 90.0 (C1′), 108.6 (CH5), 136.9 (CH6), 159.8 (C2), 171.2 (C4).

2,2′-*O*-Anhydro-5′-O-(4,4′-dimethoxytrityl)(β-D-arabinofuranosyl)uracil (51): Compound **51** was prepared as described, except that the crude product was simply purified by FC without an aqueous workup giving a 79% yield.[210] R_f = 0.33 (DCM / MeOH 10:1); ^{1}H NMR (DMSO-d_6): δ = 2.81 (dd, *J* = 7.4, 10.2, H5′) 2.95 (dd, *J* = 4.4, 10.3, H5″), 3.73 (2s, 2 -OCH_3), 4.23 (m, H4′), 4.31 (m, H3′), 5.22 (dd, *J* = 0.7, 5.7, H2′), 5.88 (d, *J* = 7.4, H5), 6.00 (d, *J* = 4.5, 3′-OH), 6.33 (d, *J* = 5.6, H1′), 6.84 (m, 4 Ar-H), 7.10-7.33 (m, 9 Ar-H), 7.96 (d, *J* = 7.5, H6). ^{13}C NMR[211] (DMSO-d_6): δ = 55.0 (-OCH_3), 62.8 (C5′), 74.7 (C3′), 85.4 (5′-OCDMT), 86.9 (4′), 88.4 (C2′), 89.8 (C1′), 108.9 (CH5), 113.2 (Ar-CH), 126.7 (Ar-CH), 127.4 (Ar-CH), 127.9 (Ar-CH), 129.4 (Ar-CH), 129.5 (Ar-CH), 135.1 (Ar-C), 135.2 (Ar-C), 136.8 (CH6), 145.9 (Ar-C), 158.1 (2s, Ar-C), 159.3 (C2), 170.9 (C4).

2,2′-*O*-Anhydro-3′-*O*-(*N*-methyl-carbamoyl)-5′-*O*-(4,4′-dimethoxytrityl)uridine (52): Compound **52** was prepared as described in 90% yield.[190] R_f = 0.26 (DCM / MeOH 95:5 with 1% TEA); ^{1}H NMR (DMSO-d_6): δ = 2.6 (d, *J* = 4.6, -NCH_3), 2.85 (dd, *J* = 7.2, 10.3, H5′), 3.00 (dd, *J* = 4.8, 10.3, H5″), 3.70 (s, -OCH_3), 4.37 (m, H4′), 5.17 (d, *J* = 2.0, H3′), 5.42 (d, *J* = 5.7, H2′), 5.89 (d, *J* = 7.5, H5), 6.37 (d, *J* = 5.7, H1′), 6.83 (m, 4 Ar-H), 7.10-7.33 (m, 9 Ar-H), 7.40 (m, -N*H*CH_3), 7.95 (d, *J* = 7.5, H6); ^{13}C NMR[211] (DMSO-d_6): δ = 27.0 (-NCH3), 55.0 (-OCH_3), 62.7 (C5′), 76.6 (C3′), 84.6 (C4′), 85.6 (-OCDMT) 86.4 (C2′), 89.9

(C1′), 109.0 (CH5), 113.2 (Ar-CH), 126.7 (Ar-CH), 127.4 (Ar-CH), 127.9 (Ar-CH), 129.4 (Ar-CH), 129.5 (Ar-CH), 135.0 (Ar-C), 135.1 (Ar-C), 136.7 (CH6), 144.5 (Ar-C), 155.1 (-O*C*ONHCH$_3$) 158.1 (2s, Ar-C), 159.2 (C2), 170.7 (C4); MALDI-TOF calc. for $C_{32}H_{32}N_3O_8$ $[M+H]^+$ 586.2, found 585.8.

***N*2′, *O*3′-Carbonyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-(methylamino)uridine (53):** Compound **53** was prepared as described in 80% yield.[190] R_f = 0.39 (DCM / MeOH 95:5 with 1% TEA); ^{1}H NMR (DMSO-d$_6$): δ = 2.85 (s, -NCH$_3$), 3.19 (dd, *J* = 4.14, 10.1, H5′), 3.38 (dd, *J* = 7.0, 10.0, H5″), 3.73 (s, -OCH$_3$), 4.21 (m, H4′), 4.51 (dd, *J* = 2.2, 8.6, H3′), 4.96 (dd, *J* = 5.2, 8.6, H2′), 5.6 (d, *J* = 8.0, H5), 5.93 (d, *J* = 2.2, H1′), 6.88 (m, 4 Ar-H), 7.18-7.42 (m, 9 Ar-H), 7.73 (d, *J* = 8.0, H6), 11.46 (s, NH); ^{13}C NMR[211] (DMSO-d$_6$): δ = 29.8 (-NCH3), 55.0 (-OCH$_3$), 63.4 (C5′), 65.9 (C3′), 76.3 (C4′), 84.9 (C2′), 85.7 (-OCDMT), 90.6 (C1′), 101.0 (CH5), 113.2 (Ar-CH), 126.8 (Ar-CH), 127.6 (Ar-CH), 127.8 (Ar-CH), 129.7 (2s, Ar-CH), 135.2 (Ar-C), 135.3 (Ar-C), 142.4 (CH6), 144.5 (Ar-C), 150.1 (C2), 156.3 (-O*C*ONHCH$_3$) 158.1 (2s, Ar-C), 163.2 (C4); MALDI-TOF calc. for $C_{32}H_{32}N_3O_8Na$ $[M+Na]^+$ 608.2, found 608.5.

2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-(methylamino)uridine (40): Compound **40** was prepared as described in 90% yield.[190] R_f = 0.27 (DCM / MeOH 95:5 with 1% TEA); ^{1}H NMR (DMSO-d$_6$): δ = 2.85 (s, -NCH$_3$), 3.19 (dd, *J* = 4.1, 10.1, H5′), 3.38 (dd, *J* = 6.8, 10.2, H5″), 3.73 (s, -OCH$_3$), 4.21 (m, H4′), 4.51 (dd, *J* = 2.1, 8.6, H3′), 4.96 (dd, *J* = 5.2, 8.6, H2′), 5.60 (dd, *J* = 1.8, 8.0, H5), 5.93 (d, *J* = 2.1, H1′), 6.88 (m, 4 Ar-H), 7.19-7.42 (m, 9 Ar-H), 7.7 (d, 8.0, H6), 11.45 (d, *J* = 1.4, NH); ^{13}C NMR[211] (DMSO-d$_6$): δ = 30.0 (-NCH3), 55.1 (-OCH$_3$), 63.5 (C5′), 66.0 (C3′), 76.4 (C4′), 85.0 (C2′), 85.8 (-OCDMT) 90.7 (C1′), 102.0 (CH5), 113.3 (Ar-CH), 126.9 (Ar-CH), 127.7 (Ar-CH), 128.0 (Ar-CH), 129.8 (2s, Ar-CH), 135.3 (Ar-C), 135.4 (Ar-C), 142.5 (CH6), 144.8 (Ar-C), 150.2 (C2), 156.4 (-O*C*ONHCH$_3$) 158.2 (2s, Ar-C), 163.3 (C4); MALDI-TOF calc. for $C_{31}H_{34}N_3O_7$ $[M+H]^+$ 560.2, found 560.0.

2′-Deoxy-2′-*S*-(4-methoxyphenylmethanethio)uridine (54): Compound **54** was prepared as described giving a yield of 82%.[212] R_f = 0.26 (DCM / MeOH 20:1); ^{1}H NMR (DMSO-d$_6$): δ = 3.32(dd, *J* = 5.2, 8.5, H2′), 3.54 (dd, *J* = 3.6, 4.8, H5′), 3.64 (d, *J* = 2.2, -SCH$_2$-), 3.72 (s, -BnOCH$_3$) 3.87 (m, H4′), 4.16 (dd, *J* = 2.0, 5.3, H3′), 5.1 (t, *J* = 5.1, 5′-OH), 5.56 (dd, *J* = 2.0,

8.1, H5), 5.64 (d, *J* = 5.3, 3′-OH), 6.04 (d, *J* = 8.6, H1′), 6.80 (doubletoid, *J* = 8.7, 2 *meta*-H as seen from thiol on benzene), 7.13 (doubletoid, *J*=8.7, 2 *ortho*-H), 7.69 (d, *J* = 8.1, H6), 11.34 (d, *J* = 1.8, NH); ^{13}C NMR (DMSO-d_6): δ = 34.0 (-SCH_2-), 51.7 (C2′), 55.0 (-OCH_3), 61.4 (C5′), 71.9 (C3′), 86.6 (C4′), 87.4 (C1′), 102.2 (CH5), 113.7 (*meta*-C as seen from thiol on benzene), 129.7 (*ipso*-C), 129.9 (*ortho*-C), 140.0 (CH6), 150.6 (*para*-C), 158.2 (C2), 162.8 (C4). LRMS calc. for $C_{17}H_{19}N_2O_6S$ $[M+H]^+$ 381.1, found 381.1

2′-Deoxy-2′-thiouridine (42): Compound **42** was prepared similarly as described.[213] **54** (1g, 2.63 mmol) was dissolved in TFA (15 mL) containing phenol (0.37g, 3.97 mmol) and heated at 100 °C overnight. The TFA was then removed under vacuum. The dark brown syrup was coevaporated twice with EtOH to remove remaining TFA. The syrup was dissolved in water and extracted 3x with diethylether. The aqueous phase was removed under vacuum giving a light yellow solid. This was taken up once more in water and the aqueous phase extracted 2x with ethyl acetate. After lyophilization of the aqueous phase an off-white foam was obtained (501 mg, 73%). R_f = 0.18 (DCM / MeOH 5:1); ^{1}H NMR (DMSO-d_6): δ = 3.50 (m, H2′), 3.55 (d, *J* = 3.8, H5′), 3.91 (dt, *J* = 1.4, 3.6, H4′), 4.06 (dt, *J* = 1.3, 5.2, H3′), 5.69 (dd, *J* = 2.1, 8.0, H5), 5.89 (d, *J* = 9.0, H1′), 7.80 (d, *J* = 8.2, H6), 11.36 (d, *J* = 1.6, NH); ^{13}C NMR (DMSO-d_6): δ = 44.8 (C2′), 61.5 (C5′), 71.9 (C3′), 86.5 (C4′), 88.8 (C1′), 102.4 (CH5), 140.4 (CH6), 151.0 (C2), 163.0 (C4). LRMS calc. for $C_9H_{11}N_2O_5S$ $[M-H]^-$ 259.0, found 259.0.

2′-Deoxy-2′-(2,4-dinitrophenylthio)uridine (43): To a cloudy mixture of **42** (150 mg, 0.577 mmol) in 50 mM NH_4OAc (3 mL) was added fluoro-2,4-dinitrobenzene (107 mg, 0.577 mmol, 73 µL) in 800 µL MeOH. After 1 min. the solution cleared and a sticky, light yellow brown solid precipitated. After an additional 30 min. of stirring the solution became cloudy once more. The supernatant was removed and centrifuged. The pellet was combined with the main precipitate and together recrystallized from glacial acetic acid. Light yellow crystals were obtained (118 mg, 45%) R_f = 0.31 (DCM / MeOH 20:1). Alternatively, to a solution of **42** (315mg, 1.21 mmol) dissolved in 0.1 M $NaHCO_3$ (6mL) was added fluoro-2,4-dinitrobenzene (315 mg, 1.70 mmol, 170µL) and shaken vigorously. A yellow precipitate was obtained immediately. The aqueous phase was extracted with ethyl acetate. The organic phase containing the yellow precipitate was removed under vacuum. The yellow solid was taken up in 20 mL MeOH and 250 mL DCM were added to precipitate. This was left overnight at 4 °C.

The yellow precipitate was filtered and dried giving a yellow solid shown to be nearly pure by TLC (500 mg, quant.) R_f = 0.31 (DCM / MeOH 10:1). ^{1}H NMR (DMSO-d_6): δ = 3.67 (m, H5′), 3.99 (dd, *J* = 3.4, 5.9, H4′), 4.42 (dd, *J* = 5.7, 7.9, H2′), 4.50 (dt, *J* = 2.5, 5.8, H3′), 5.24 (t, *J* = 5.2, 5′-OH), 5.72 (dd, *J* = 2.1, 8.1, H5), 6.05 (d, *J* = 5.9, 3-OH), 6.13 (d, *J* = 8.0, H1′) 7.84 (m, H6, *ortho*-H as seen from thiol on dinitrobenzene), 8.33 (dd, *J* = 2.6, 9.0, *meta*-H), 8.81 (d, *J* = 2.5, *meta*-H between dinitro groups), 11.23 (d, *J* = 1.9, NH); ^{13}C NMR (DMSO-d_6): δ = 52.6 (C2′), 61.1 (C5′), 70.9 (C3′), 86.9 (C4′, C1′), 102.9 (CH5), 121.1 (*meta*-C between dinitro groups), 126.6 (*meta*-C), 130.3 (*ortho*-C), 139.9 (CH6), 142.4 (*ipso*-C), 144.5 (*para*-C-NO_2), 146.5 (*ortho*-C-NO_2), 150.3 (C2), 162.7 (C4). HRMS calc. for $C_{15}H_{15}N_4O_9S$ $[M+H]^+$ 427.0560, found 427.0551.

2′-Deoxy-2′-(2,4-dinitrophenylthio)-5′-*O*-(4,4′-dimethoxytrityl)uridine (44): To a solution of **43** (500 mg, 1.17 mmol) in pyridine (15 mL) was added 4,4′-dimethoxytrityl chloride (558 mg, 1.64 mmol) in two portions over 2 hours. The solution was stirred overnight at rt. Methanol was then added and stirred 30 min. before removing the solvent under vacuum. The solid was purified by FC (DCM / MeOH 40:1 to 10:1, with 5% TEA). A pale yellow solid was obtained (290 mg, 33%). An additional 311 mg **43** was recovered. R_f = 0.38 (5% MeOH in DCM with 1% TEA). ^{1}H NMR (CD_3DN): δ = 3.42 (d, *J* = 3.4, H5′), 3.76 (s, 2 -OCH_3), 4.12 (m, H4′), 4.27 (t, *J* = 6.4, H2′), 4.72 (m, H3′), 5.47 (d, *J* = 8.1, H5), 6.11 (d, *J* = 6.8, H1′), 6.88 (d, *J* = 8.8, 4 Ar-H), 7.21-7.38 (m, 7 Ar-H), 7.42-7.49 (m, 2 Ar-H), 7.61 (d, *J* = 8.2, H6), 7.68 (d, *J* = 9.0, *ortho*-H as seen from thiol), 8.21 (dd, *J* = 2.5, 8.9, *meta*-H), 8.83 (d, *J* = 2.4, *meta*-H between dinitro groups); ^{13}C NMR (CD_3DN): δ = 54.8 (C2′), 55.9 (2 –OCH_3), 63.9 (C5′), 71.8 (C3′), 85.7 (C4′), 87.7 (-OCDMT), 88.9 (C1′), 103.7 (CH5), 114.2 (Ar-CH), 122.2 (*meta*-C between dinitro groups), 127.8 (Ar-CH), 128.0 (Ar-CH), 129.0 (2s, Ar-CH), 131.1 (Ar-CH), 136.4 (Ar-C), 136.5 (Ar-C), 140.7 (CH6), 143.9 (*ipso*-C), 145.65 (Ar-C), 145.72 (*para*-C-NO_2), 147.7 (*ortho*-C-NO_2), 151.2 (C2), 159.7 (Ar-C), 163.6 (C4). HRMS calc. for $C_{36}H_{31}N_4O_{11}S$ $[M-H]^-$ 727.1709, found 727.1711.

2′-Deoxy-2′-(2,4-dinitrophenylthio)-5′-*O*-(4,4′-dimethoxytrityl)uridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite) (45): To a solution of **44** (85 mg, 0.117 mmol) in ACN (1 mL) containing molecular sieves was added DCI activator (1mL, 0.25 M in ACN) followed by **48** (70 mg, 0.233 mmol, 74 µL). After 2 hours stirring at rt the solution was taken up in DCM and extracted 3x with 5% $NaHCO_3$, once with brine and then

dried over $MgSO_4$ and the solvent reduced under vacuum to several mL. This volume was slowly added under vigorous stirring to 250 mL pentane forming a pale yellow precipitate. After storage at -20 °C overnight the organic phase was decanted and the sticky precipitate dried under vacuum giving a pale yellow foam (71 mg, 65%). R_f = 0.5 (5% MeOH in DCM with 1% TEA); ^{31}P NMR (CD_3CN): δ = 149.9, 150.9. HRMS calc. for $C_{45}H_{50}N_6O_{12}PS$ $[M+H]^+$ 929.2946, found 929.2941.

***N*^{4}-(2-aminoethyl)cytidine (37):** Compound **37** was prepared as described with the following modifications.[173] To cytidine (100 mg, 0.4 mmol) in a 2 mL PP screw top reaction vial was added sodium bisulfite (86 mg, 0.8 mmol), and ethylene diamine (74 mg, 1.2 mmol) and filled partially with water and mixed until dissolved. The pH was adjusted with HCl to 7.0-7.5 and the total volume brought to 2 mL. The mixture was heated at 80 °C overnight. Complete conversion was observed with TLC and no Uridine or other byproducts were observed. The pH was adjusted to 9.0 with NaOH and left standing for 1 hour. Purification was done using Bio-Gel P-2 gel (fine, Bio-Rad) with 50 mM NH_4HCO_3. A second FC with normal silica gel was performed (MeOH / 30% NH_4OH solution 9:1). Violatiles were removed under vacuum and remaining water lyophilized to give a white powder (107 mg, 91%). R_f = 0.24 (MeOH/NH_4OH 4:1). ^{1}H NMR (D_2O/CD_3OD 10:1): δ = 3.04 (t, *J* = 5.9, -NHC*H*$_2$CH$_2$NH$_2$), 3.58 (t, *J* = 5.9, -NHCH$_2$C*H*$_2$NH$_2$), 3.79 (dd, *J* = 4.2, 12.8, H5′), 3.90 (dd, *J* = 2.8, 12.7, H5″), 4.07-4.13 (m, H4′), 4.18 (t, *J* = 5.5, H3′) 4.27 (dd, *J* = 4.2, 5.1, H2′), 5.89 (d, *J* = 4.2, H1′) 6.01 (d, *J* = 7.6, H5), 7.78 (d, *J* = 7.6, H6); ^{13}C NMR (D_2O/CD_3OD 10:1): δ = 41.1 (-CH$_2$-) ,41.2 (-CH$_2$-) 61.7 (C5′), 70.4 (C3′), 75.0 (C2′), 84.9 (C4′), 91.1 (C1′), 98.2 (CH5), 141.2 (CH6), 158.8 (C2), 165.7 (C4). MALDI-TOF calc. for $C_{11}H_{19}N_4O_5$ $[M+H]^+$ 287.1, found 286.8.

***N*^{4}-(4-(4-(dimethylaminophenylazo)benzamido)ethylcytidine (38):** *N*^{4}-(2-aminoethyl)cytidine **37** (87 mg, 0.3 mmol) was placed together with 4-(4-(dimethylaminophenylazo)benzoic acid NHS ester (134 mg, 0.37 mmol) in a 2 mL PP screw top reaction vial. 1.5 mL DMSO was added to dissolve the solids. Then DIPEA (117 mg, 0.9 mmol) was added and mixed well. The reaction was left at rt for 6 hours. The reaction mixture was first flushed over a short silica column with DCM / MeOH 4:1 to remove DMSO. The dried eluent containing product was then purified by FC (DCM / MeOH / NH_4OH 9:2:0.5) to give a red solid (102 mg, 63%). R_f = 0.14 (DCM/MeOH/NH_4OH 9:2:0.5). ^{1}H NMR (CD_3OD): δ = 3.08 (s, -N(CH$_3$)$_2$), 3.62 (m, 2 -CH$_2$-), 3.74 (dd, *J* = 3.2, 12.3, H5′), 3.88 (dd, *J* = 2.5, 12.3,

H5″), 4.03 (m, H4′), 4.15 (m, H3′, H2′), 5.89 (d, *J* = 2.9, H1′), 5.92 (d, *J* = 7.6, H5), 6.80 (d, *J* = 9.3, 2 Ar-H), 7.81 (d, *J* = 3.7, 2 Ar-H), 7.84 (d, *J* = 4.2, 2 Ar-H), 7.95 (d, *J* = 8.6, 2-Ar-H), 7.99 (d, *J* = 7.6, H6); ^{13}C NMR (CD_3OD): δ = 40.4 (2 CH_3), 40.8 (-CH_2-) ,41.1 (-CH_2-) 62.0 (C5′), 70.8 (C3′), 76.1 (C2′), 85.9 (C4′), 92.0 (C1′), 97.0 (CH5), 112.6 (2 Ar-CH), 123.0 (2 Ar-CH), 126.4 (2 Ar-CH), 129.4 (2 Ar-CH), 135.4 (Ar-C), 141.9 (CH6), 144.7 (Ar-C), 154.6 (Ar-C), 156.4 (Ar-C), 158.1 (C2), 165.5 (C4), 169.7 (NHCO). MALDI-TOF calc. for $C_{26}H_{32}N_7O_6$ $[M+H]^+$ 538.2, found 537.9.

***N*^{4}-(4-(4-(dimethylaminophenylazo)benzamido)ethylcytidine-3′(2′),5′-bisphosphate (39):** The 3′(2′), 5′-bisphosphate **39** was prepared similarly to the literature.[175]

N^4-(4-(4-(dimethylaminophenylazo)benzamido)ethylcytidine **38** (18mg, 33.5 μmol) was placed in a 2 mL PP screw top vial and 100 μL pyrophosphoryl chloride was added under argon at -10 °C. The mixture was dissolved by vortexing briefly and the reaction allowed to proceed for 3.5 hours at -15 to -10 °C. The reaction was quenched over ice and then with 1 M TEAB buffer and the aqueous buffer removed under vacuum. The red residue was dissolved in MeOH and evaporated several times to remove remaining buffer. The residue was purified on a short C_{18} silica gel column (7 x 2 cm) using 0.1 M TEAA (buffer A) and ACN (B) (as described for HPLC). The sample was loading at 0% B and rinsed with an additional 2 volumes buffer A. A gradient was then applied from 20-50% B with product eluting between 30-50% B. Buffer was removed under vacuum to give a red solid (10 mg, 43%, 2:1 3′,5′ / 2′,5′). ^{31}P NMR (D_2O): δ (2′,5′-bisphosphate) = 2.93 (s, 2′-P), 3.35 (s, 5′-P); δ (3′,5′-bisphosphate) = 3.22 (s, 5′-OPO_3), 3.61 (s, 5′-P). MALDI-TOF calc. for $C_{26}H_{32}N_7O_{12}P_2$ $[M-H]^-$ 696.17, found 696.18.

5.2.2 RNA Synthesis

5.2.2.1 General Protocols

Automated RNA synthesis: Syntheses were performed using an Applied Biosystems 3400 DNA Synthesizer (Foster City, CA USA) using ports 5-8 for the RNA monomer phosphoramidites. Monomers were placed on the machine and filled with dry ACN directly from the machine to the desired volume using the timed filling command. Only standard couplings were performed on the synthesizer. The 2′-TOM protected β-cyanoethyl phosphoramidite monomers were used for all RNA synthesized. BMT was used as activator at

a concentration of 0.25 M in dry ACN. Deblock solution contained 3% TCA in DCM. Cap A contained THF/ pyridine / acetanhydride in a 8:1:1 ratio. Cap B contained 16% 1-methylimidazole in THF. The oxidation reagent consisted of 0.02 M iodine in THF / pyridine / water in a ratio of 7:2:1. Standard RNA synthesis protocols from the manufacturer were used except that coupling times were set to 6 min.

General protocol for manual RNA synthesis (protocol A): To a 1.5 mL PP vial the desired amount of cpg was added. If necessary, the 5′-DMT group was removed by reacting with deblocking solution for 1 min. and then rinsed extensively with ACN to remove traces of TCA before continuing with the coupling step. After removal of the 5′-DMT group 20-50 molar equivalents of the phosphoramidite to be coupled were added to the previously dried cpg. This was placed on a high vacuum line for at least 2 hours and then removed under argon. The appropriate volume of 0.3 M BMT activator solution was added directly onto the phosphoramidite / cpg so as to obtain a final 0.05 M concentration of the phosphoramidite. In the case of a liquid phosphoramidite (e.g. 5′-phosphorylation reagent) the reagent was added directly to the BMT solution / cpg using a pipette tip (scraping of an appropriate amount of thick reagent from bottle using tip). Reactions were allowed to proceed for 5-10 min. while being shaken. The cpg was rinsed once with dry ACN. If necessary, 500 µL each Cap A and Cap B were added, mixed, and allowed to react for one min. The excess capping reagents were removed and the cpg rinsed once with ACN. Oxidation reagent was added in excess and allowed to react for one min. The cpg was rinsed 2-3 times with excess ACN until the iodine color was no longer seen and then allowed to dry on a high vacuum line. Unless otherwise noted the cpg was stored at -20 °C until further needed.

General protocols for cleavage of 2′-TOM-protected RNA from cpg and base protecting group removal

Protocol B: The cpg was transferred to a 1.5 mL PP vial and 2 M dry methanolic ammonia was added. The vial was shaken for 1 hour at rt. The supernatant was transferred to a new vial and the cpg rinsed with MeOH and also collected. The combined phases were evaporated under reduced pressure to dryness. Methylamine solution MA, 1:1 mixture of 40% aqueous methylamine and 33% ethanolic methylamine) or AMA (1:1 mix of concentrated ammonium hydroxide and 40% aqueous methylamine) was added to the residue and vortexed to dissolve.

This was allowed to react for 3 hours (MA) or 2 hours (AMA) before being evaporated under reduced pressure to dryness.

Protocol C: The cpg was transferred to a 1.5 mL PP vial and MA was added. The cpg was incubated at 35 °C for 6 hours or overnight at rt. The supernatant was transferred to a new vial and the cpg rinsed with water and also collected. The combined phases were evaporated under reduced pressure to dryness.

Protocol D: The cpg was transferred to a 1.5 mL PP vial and AMA solution was added. The vial was shaken for 2 hours at rt. The supernatant was transferred to a new vial and the cpg rinsed with water and also collected. The combined phases were evaporated under reduced pressure to dryness.

General protocol for removal of 2′-TOM-protecting groups from RNA

Protocol E: To the dried RNA residue was added one volume DMSO to dissolve and then 1.25 volumes triethylamine trihydrofluoride (Aldrich, # 344648). The mixture was heated for 1.5 hours at 65 °C. After cooling, 0.25 volumes 3 M sodium acetate were added, vortexed, and 2.5 volumes EtOH or 1.5 volumes IPA added and vortexed. After leaving overnight at -20 °C the RNA was centrifuged for 30 min. at 12,100 g. The supernatant was removed and the pellet washed with 70% EtOH. After another 30 min. centrifugation the supernatant was removed and the pellet dried on air to remove residual EtOH.

Protocol F: To the dried RNA residue was added one volume of 1 M TBAF in THF. The residue was heated at 50 °C for 10 min. to dissolve using ultrasound if necessary to break up any particles. The mixture was incubated at 35 °C for at least 6 hours. One volume of 1 M Tris-HCl, pH 7.4 was added and vortexed well. After leaving at rt for at least 30 min. the THF was evaporated under reduced pressure and the RNA precipitated as in general protocol E.

5.2.2.2 Syntheses

Pre-mir-142 FAM-CCAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′, 33mer (2): A 0.2 μmol automated synthesis was carried out until the second from last cytidine. 1/3 of the cpg was set aside for synthesis of **1** (below). The remaining 2/3 cpg was replaced on the synthesizer and coupling of the remaining cytidine carried out (33 bases, DMT-off). Coupling of 6-FAM phosphoramidite (5 mg, 5.9 μmol) was carried out according

to protocol A and then deprotected according to protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 20% B in 40 min.: t_R = 32 min. Yield: 32%. MALDI-TOF calc. $[M-H]^-$ 11127.1, found 11141.6.

Pre-mir-142 5′-(6)FAM-CAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′, 32 mer (1): 1/3 of the cpg from 2 (32 mer) was treated to remove the DMT group as in protocol A. The cpg was coupled with (6)FAM phosphoramidite (3 mg, 3.6 µmol) according to general protocol A and then deprotected according to protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 20% B in 40 min.: t_R = 32 min. Yield: 35%. MALDI-TOF calc. $[M-H]^-$ 10843.5, found 10843.7.

Pre-mir-142 5′-PO_3-AGGGUGUAGUGUUUCCUACUUUAUGGdU-DABCYL-3′, 27 mer (3): A 0.2 µmol USIIPS was coupled with dU-DABCYL (7 mg, 6.1µmol) according to general protocol A. Automated RNA synthesis was then carried out (DMT-off). The 5′-phosphate was coupled according to protocol A using the commercial 5′-phosphorylating reagent (~10 µL). Deprotection was done according to protocol B, followed by protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 34 min. Yield: 63% (not based on DABCYL-dU loading). MALDI-TOF calc. $[M-H]^-$ 9088.9, found 9092.5.

Pre-mir-19b-2 5′-Cy3-CAGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 32 mer (5): A 0.2 µmol automated synthesis was carried out until the second from last cytidine. 1/3 of the cpg was set aside for synthesis of **4** (below). The remaining 2/3 cpg was replaced on the synthesizer and coupling of the remaining cytidine carried out (32 bases, DMT-off). Coupling of Cy3 phosphoramidite (5 mg, 5.2 µmol) was carried out according to protocol A with the MMT protecting group being removed at the end of the synthesis as in protocol A. The support was then deprotected according to protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 32 min. Yield: 36%. MALDI-TOF calc. $[M-H]^-$ 10672.0, found 10676.9.

Pre-mir-19b-2 5′-Cy3-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 31 mer (4): 1/3 of the cpg from **5** (31 mer) was treated to remove the DMT group as in protocol

A. The cpg was coupled with Cy3 phosphoramidite (3mg, 3.1 µmol) according to protocol A with the MMT protecting group being removed at the end of the synthesis as in protocol A. The support was then deprotected according to protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 32 min. Yield: 42%. MALDI-TOF calc. $[M-H]^-$ 10366.8, found 10376.3.

Pre-mir-19b-2 5′-(6)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 31 mer (6): A 0.2 µmol automated synthesis was carried out (31 bases, DMT-off). Coupling of (6)TMR phosphoramidite (5 mg, 6.1 µmol) was carried out according to protocol A with a coupling time of 1 hour. A final capping step for 5 min. was done as in protocol A. The support was then deprotected with TBA/H_2O 1:3 at 65 °C for 3 hours. The supernatant was transferred to a new vial and the cpg rinsed with H_2O and also collected. The combined phases were evaporated under reduced pressure to dryness. The residue was then deprotected according to protocol F. Yield not determined. MALDI-TOF calc. $[M-H]^-$ 10535.0, found 10519.2, 10561.3 (+Ac), 10605.5(+ 2 Ac), 10648.8(+3 Ac), etc.

Pre-mir-19b-2 5′-PO_3-UGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′, 35 mer (7): A 0.2 µmol USIIPS was coupled with dU-DABCYL (7 mg, 6.1 µmol) according to general protocol A. Automated RNA synthesis was then carried out (DMT-off). The 5′-phosphate was coupled according to protocol A using the commercial 5′-phosphorylating reagent (~10 µL). Deprotection was done according to protocol B, followed by protocol D, followed by protocol E. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 35 min. Yield: 63%. MALDI-TOF calc. $[M-H]^-$ 11673.5, found 11679.6.

Pre-bantam 5′-(6)FAM-CCGGUUUUCGAUUUGGUUUGACUGUUUUUC-OH-3′, 30 mer (8): A 0.2 µmol automated synthesis was carried out (30 bases, DMT-off). Coupling of (6)FAM phosphoramidite (5 mg, 5.9 µmol) was carried out according to protocol A and then deprotected according to protocol C, followed by protocol F. HPLC gradient 0% B for 4 min. to 20% B in 40 min.: t_R = 35 min. Yield: 27%. MALDI-TOF calc. $[M-H]^-$ 9974.5, found 9967.4.

Pre-bantam 5′-PO$_3$-AUACAAGUGAGAUCAUUUUGAAAGCUGAUdU-DABCYL-3′, 30 mer (9): A 0.2 µmol USIIPS was coupled with dU-DABCYL (7 mg, 6.1 µmol) according to general protocol A. Automated RNA synthesis was then carried out (DMT-off). The 5′-phosphate was coupled according to protocol A using the commercial 5′-phosphorylating reagent (~10 µL). Deprotection was done according to protocol B, followed by protocol C, followed by protocol F. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 38 min. Yield: 53%. MALDI-TOF calc. $[M-H]^-$ 10090.5, found 10084.2.

Dicer siRNA-1 5′-PO$_3$-UCCAGAGCUGCUUCAAGCAGU-OH-3' (55): A 0.2 µmol automated synthesis was carried out (21 bases, DMT-off). Coupling of bis-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite **47** (~ 10 µL) was carried out according to protocol A and then deprotected according to protocol C, followed by protocol E. HPLC gradient 0% B for 4 min. to 15% B in 40 min.: t_R = 31 min. Yield: 23%. MALDI-TOF calc. $[M-H]^-$ 6751.2, found 6755.1.

Dicer siRNA-2 5′-PO$_3$-UGCUUGAAGCAGCUCUGGAUC-OH-3' (56): A 0.2 µmol automated synthesis was carried out (21 bases, DMT-off). Coupling of bis-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite **f** (~ 10 µL) was carried out according to protocol A and then deprotected according to protocol C, followed by protocol E. HPLC gradient 3% B for 4 min. to 12% B in 40 min.: t_R = 32 min. Yield: 33%. MALDI-TOF calc. $[M-H]^-$ 6768.2, found 6772.5.

Control siRNA-1 5′-PO$_3$-CUUUAAGCUCCCUGAGCGUUU-OH-3' (57): A 0.2 µmol automated synthesis was carried out (21 bases, DMT-off). Coupling of bis-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite **47** (~ 10 µL) was carried out according to protocol A and then deprotected according to protocol C, followed by protocol E. HPLC gradient 3% B for 4 min. to 12% B in 40 min.: t_R = 30 min. Yield: 41%. MALDI-TOF calc. $[M-H]^-$ 6666.2, found 6671.4.

Control siRNA-2 5′-PO$_3$-ACGCUCAGGGAGCUUAAAGUG-OH-3' (58): A 0.2 µmol automated synthesis was carried out (21 bases, DMT-off). Coupling of bis-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite **47** (~ 10 µL) was carried out according to protocol A and

then deprotected according to protocol C, followed by protocol E. HPLC gradient 3% B for 4 min. to 12% B in 40 min.: t_R = 31 min. Yield: 26%. MALDI-TOF calc. $[M-H]^-$ 6854.2, found 6860.1.

5′-DMT-$U_HU_{SH}U_HU_{SH}$G-OH-3′ (46): 44 nmol (1.1 mg, 40μmol/g) G cpg was placed in a 1.5 mL PP reaction vial. This was alternatively coupled according to protocol A with first **45** (2 mg, 2.2 μmol) and then **41** (2 mg, 2.7 μmol) using 0.25 M DCI activator. The final DMT group was left on. The now yellow colored cpg was treated according to protocol C. Yield not determined. MALDI-TOF calc. $[M-H]^-$ 1809.4, found 1810.5.

5.3 Molecular and Cell Biology

5.3.1 Materials and Methods

General materials and methods for handling nucleic acids were adopted from *Molecular cloning : a laboratory manual, 3rd. ed., vol.1-3*.[214] If not otherwise mentioned any cellular and molecular biology methods were taken from this source. Any differences to these procedures are noted below. As stated previously, all plastic materials purchased were certified Pyrogen / DNAse / RNAse free when possible. All plastic reaction vials were obtained from Sarstedt (Nümbrecht, Germany), Cotech (Berlin, Germany) or Carl Roth (Karslruhe, Germany). Pipette tips were purchased from either Cotech or Carl Roth. (5/6)FAM NHS ester, and DABCYL NHS ester were purchased from EMP Biotech (Buch, Germany). The 5′-FAM-36 mer (**59)**, 3′-DABCYL-37 mer (**60**), 3′-unlabeled 36 mer (**61**) for beacons **10** and **11**, and 5′-TMR-31 mer (**6**) for beacon **16** were purchased from IBA (Göttingen, Germany). Additionally, 5′-amino-ApG and 5′-(5/6)FAM-ApG, 5′-(5/6)TMR-ApG transcription starters were obtained from IBA. RNA size ladder was purchased from Ambion. Solutions and pipette tips (if not already certified Pyrogen/DNAse/RNAse free) for cell culture use were autoclaved. RNAse inhibitor was purchased from either Promega (RNAsin) or Fermentas (Ribolock). D-MEM with phenol red (#31966.021), trypsin-EDTA solution (0.25% with 1 mM EDTA, #25200-056), and antibiotics (#1548-140) were purchased from Invitrogen. D-MEM without phenol red (#F 0475) and trypsin (0.25%, #L 2123) were purchased from Biochrom (Berlin, Germany). FCS was obtained from either Invitrogen or Biochrom (#S 0615). The HEK 293 cell line was obtained from Invitrogen. The laminar flow hood was from BDK Luft- und Reinraumtechnik (Sonnenbühl-Genkingen, Germany), CO_2 incubator from Sanyo (Osaka, Japan), ultrasound cell disruptor 450 (cooled) from Branson

(Danbury, CT, USA), and the autoclave a Systec 3150 ELV (Wettenberg, Germany). For Western blot analysis PAGE electrophoresis a semi-dry transfer apparatus from Bio-Rad was used. For microscopy work petri-dishes with glass bottoms (#P35G-1.5-14-C) from MatTek (Ashland, MA, USA) were used.

RNA precipitation: RNA was precipitated by adding 1/10 volume of either 3 M NaOAc, pH 5.2 or 5 M NH_4OAc, pH 7.5 to the RNA-containing solution. Where necessary, for extremely dilute solutions for analytical purposes either linear polyacrylamide (10-20 µg / mL, for > 20 mers)[215] or glycogen (GlycoBlue™, Ambion for > 6 mers) was added after addition of salt. After mixing well 3-6 volumes EtOH or 1.5-4 volumes IPA were added, mixed, and incubated overnight at -20 °C. In cases where overnight incubation was not feasible, samples were frozen in liquid nitrogen and cooled at -20 °C an additional hour before centrifuging at 12,100 g for 30 min. at 4 °C. The supernatant was removed and the pellet washed with 80% cold EtOH and centrifuged again at 12,100 g for 30 min. at 4 °C. The supernatant was removed, the pellet dried to remove excess EtOH and then taken up in 1 mM ammonium citrate, pH 6.4. Samples for long-term storage were left in the precipitation salt / alcohol solution at -80 °C.

PCI extraction: Phenol / chloroform / isoamyl alcohol (25:24:1) equilibrated with TE buffer pH 7.5-8.0 (PCI) (Sigma-Aldrich or Carl Roth) was used to extract RNA from enzymatic reaction solutions according to standard procedure.[214] Back extraction of the organic phase was carried out to ensure optimal recovery of RNA. The RNA was subsequently precipitated.

CIP reaction: Calf intestinal phosphatase from New England BioLabs (NEB, #M0290) was used according to the manufacturer's instructions to remove 5′- and 3′-phosphate groups from RNA. Briefly, the reaction buffer was composed of 50 mM Tris-HCl, 100 mM NaCl 10 mM $MgCl_2$, 1 mM DTT, pH 7.9. At least 4 U enzyme was used per 1 nmol RNA. 0.3-1 U / µL RNAse inhibitor was also added and reactions incubated at 37 °C for at least 1 hour. The RNA was purified by PCI extraction and precipitation before further use.

PNK reaction: T4 polynucleotide kinase from NEB (#M0201) was used to phosphorylate the 5′-terminus of RNA generally according to the manufacturer's instructions. Briefly, the reaction buffer was composed of 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP,

pH 7.8. At least 15 U enzyme was used per 1 nmol RNA. 0.3-1 U/µL RNAse inhibitor was also added and reactions incubated at 37 °C for at least 1 hour. The RNA was purified by PCI extraction and precipitation before further use.

Electrophoresis: Polyacrylamide gel electrophoresis (PAGE) analysis was done using either native or denaturing (8 M urea) gels containing a 15% or 20 % running and 4% stacking gels (bis:acrylamide, 1:19). A mini-Protean II system from Bio-Rad (Hercules, CA, USA) was used. A constant voltage of 250 V in TBE buffer, pH 8.3, was applied. For native gels 6x sucrose loading buffer (18 mM EDTA, 0.05% bromophenol blue, 40% sucrose) was used. For denaturing gels 1x formamide loading buffer (90% formamide, 18 mM EDTA, and 0.05% bromophenol blue) was added to samples at a minimum ratio of 1:1 and denatured at 95 °C for 5 min. and cooled on ice before loading. Either SYBR Green II or SYBR Gold (Invitrogen, 1:10,000 dilution in 1 TBE buffer) was used for staining gels. Gels were viewed under UV light (300 nm) and photographed using an Olympus C-5060 5 megapixel CCD camera (Tokyo, Japan)

Gel Elution: Desired bands from PAGE gels were cut out and extracted using the 'crush and soak' method. RNAse inhibitor was added at 0.1 U / µL to prevent possible degradation. These solutions were PCI extracted, butanol extracted if necessary to reduce the volume, and precipitated. For analytical purposes RNA was generally precipitated using a carrier (linear polyacrylamide or GlycoBlue^{TM} glycogen / Ambion).

T_m determination for beacon 10: The T_m for the beacon was measured in buffer containing 250 mM NaCl, 20 mM Tris–HCl, pH 7.4, and 2.5 $MgCl_2$. Heating and cooling rates of 1 °C / min. were used. The sample was first renatured by heating to 85 °C and then cooling to 5 °C. A T_m = 59 °C was then calculated as the average of three measurements from the maximums of the first derivatives.

General ligation protocol for making beacons with synthetic RNA strands (general protocol G): Ligations were performed using T4 RNA ligase 1 from NEB (#M0204S). The strands to be ligated were added to a 1.5 mL PP reaction vial. The supplied reaction buffer gave final concentrations of 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH

7.8 and was added together with 0.3-0.8 U/µL RNAse inhibitor and 0.3-0.8 U/µL T4 RNA ligase I. The final concentration of ss RNA was 5-40 µM. The RNA was purified by PCI extraction and precipitation before further use.

General protocol for *in vitro* transcription of RNA (general protocol H):

A Promega T7 RiboMAX Express *in vitro* transcription kit (#1320) was used for all *in vitro* transcription reactions. DNA templates were purchased with double HPLC purification. The pre-let-7 template and T7 promoter sequence were purchased from Proligo (Germany). All other templates were purchased from Biotez (Buch, Germany). The T7-promoter sequence (bold) used in all DNA templates was 5′-DNATemplateSequence-**TATAGTGAGTCGTATTA**-3′. The complementary oligo used to create the ds T7 promoter region was 5′-GGTAATACGACTCACTATAG-3′. Transcription was done according to the manufacturer taking advantage of DNAse degradation of template at the end of the reaction according to the manufacturer. An extended transcription reaction time of 2-4 hours was performed. The RNA was subsequently dephosphorylated with alkaline phosphatase and rephosphorylated with polynucleotide kinase, if necessary.

General procedure for assay: Pipetting errors were minimized by combining beacon and buffer salts at a concentration slightly above the final concentration. For example, testing of potential pre-miRNA binders was performed as follows: The beacon was renatured in Dicer reaction buffer (20 mM Tris-HCl, pH 6.8, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) at a concentration of 25 nM and buffer constituents 1.25x the final strength. Then 32 µL of this RNA solution was combined with 4 µL test substance in one well in a 384-well microtiter plate and covered with Parafilm. After 30 min. incubation at rt the plate was placed on ice for 10 min. before adding 4 µL Dicer enzyme at the desired strength (e.g. 40x diluted gave a total of 0.1 U enzyme in one well. Dicer enzyme dilution buffer consisted of 100 mM NaCl, 20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM DTT, 0.1% Triton X-100, and 10% glycerol). The complete experiment was then placed in the plate reader (BMG) and the fluorescence increase measured every minute for 4 hours. Experiments were performed at least in duplicate when not triplicate and the average taken.

5.3.2 Beacons *via* Ligation

FAM pre-let-7

5′-(6)FAM-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAU ACUAUACAAUGUGCUAGCUUUCUUUGCUdC-DABCYL-3′ (10): The RNA strands **59** and **60** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 37 min. Yield: 68%. MALDI-TOF calc. $[M-H]^-$ 24411.3, found 24431.6.

FAM-only pre-let-7

5′-(6)FAM-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAU ACUAUACAAUGUGCUAGCUUUCUUUGCU-OH-3′ (11): The RNA strands **59** and **61** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 25 min. Yield: 70%. MALDI-TOF calc. $[M-H]^-$ 23696.0, found 23697.5.

FAM pre-mir-142, 60 mer

5′-(6)FAM-CCAUAAAGUAGAAAGCACUACUAACAGCACUGGAGGGUGUAGUG UUUCCUACUUUAUGGdU-DABCYL-3′ (12): The RNA strands **2** and **3** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 37 min. Yield: 27 %. MALDI-TOF calc. $[M-H]^-$ 20199.0, found 20205.9.

FAM pre-mir-142, 59 mer

5′-(6)FAM-CAUAAAGUAGAAAGCACUACUAACAGCACUGGAGGGUGUAGUGU UUCCUACUUUAUGGdU-DABCYL-3′ (13): The RNA strands **1** and **3** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 31 min. Yield: 32%. MALDI-TOF calc. $[M-H]^-$ 19893.8, found 19904.5.

Cy3 pre-mir-19b-2, 67 mer

5′-Cy3-CAGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGU GCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (14): The RNA strands **5** and **7** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 36 min. Yield: 28%. MALDI-TOF calc. $[M-H]^-$ 22328.5, found 22326.5.

Cy3 pre-mir-19b-2, 66mer

5′-Cy3-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (15): The RNA strands **4** and **7** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 36 min. Yield: 24%. MALDI-TOF calc. $[M-H]^-$ 22020.2, found 22017.7.

TMR pre-mir-19b-2, 66 mer self-made

5′-(6)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (16): The RNA strands **6** and **7** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 36 min. Yield not determined. MALDI-TOF calc. $[M-H]^-$ 22191.5, found 22201.4 (additional peaks for +Ac, +2Ac, +3Ac, +4Ac, etc.).

TMR pre-mir-19b-2, 66 mer made with IBA TMR 5′-strand

5′-(5)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (16): The RNA strands **6** (IBA) and **7** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 35 min. Yield: 37%. MALDI-TOF calc. $[M-H]^-$ 20107.3, found 22097.5.

FAM pre-bantam

5'-(6)FAM-CCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGAUCAUUUUGAAAGCUGAUdU-DABCYL-3′ (17): The RNA strands **8** and **9** were ligated according to protocol G. HPLC gradient 0% B for 4 min. to 40% B in 40 min.: t_R = 35 min. Yield: 27%. MALDI-TOF calc. $[M-H]^-$ 20048.0, found 20038.3.

5.3.3 Beacons and RNA using *in vitro* Transcription

Pre-let-7 unlabeled

5′-PO_3-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAUACUAUACAAUGUGCUAGCUUUCUUUGCU-OH-3′ (62): An *in* vitro transcription was carried out according to protocol H. The following DNA template was used:

5′-AGCAAAGAAAGCTAGCACATTGTATAGTATGATGTGTAATTACTACTATACTTCCT ACTACCTCAATTTGCC**TATAGTGAGTCGTATTA**-3′. The 5′-triphosphate was removed with CIP and a single 5′-phosphate added using PNK as described. HPLC gradient 3% B for 4 min. to 20% B in 40 min.: t_R = 32 min. MALDI-TOF calc. $[M-H]^-$ 23121.4, found 23112.1.

FAM-only pre-bantam using amino-ApG and FAM NHS-ester

5′-(5/6)FAM-AGCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGA UCAUUUUGAAAGCUGAUU-OH-3′ (30): An *in* vitro transcription was carried out according to protocol H with the addition of 1 mM 5′-amino-ApG transcription starter in a final volume of 20 µL. The following DNA template was used: 5′-AATCAGCTTTCAAAATGATCTCACTTGTATGAAAAACAGTCAAACCAAATCGAA AACCGC**TATAGTGAGTCGTATTA**-3′. The crude product was then taken up in 57 µL 100 mM sodium borate buffer, pH 8.6. (5/6)FAM NHS ester (~500 µg, 1 µmol) was dissolved in 14 µL DMSO. This was added to the RNA. An additional 29 µL water was used to rinse and carry over any remaining FAM to the RNA. The grainy yellow mixture was vortexed well and allowed to react overnight at rt in the dark while being shaken. The reaction mixture was precipitated with NaOAc and IPA. HPLC gradient 3% B for 4 min. to 15% B in 40 min.: t_R = 38 / 41 min. both isomers, only 41 min. kept. Yield: 0.37 nmol, 28%. MALDI-TOF calc. $[M-H]^-$ 20014.7, found 20002.4.

FAM-morpholino Dabcyl pre-bantam

5′-(5/6)FAM-AGCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGA UCAUUUUGAAAGCUGAUU-DABCYL-3′ (31): The procedure by Beuvink *et al* was adapted for use here.[172] The FAM-labeled product **30** (6 µg, 0.3 nmol) was dissolved in 9 µL water and to this was added 1 µL freshly prepared 100 mM sodium periodate followed by 1 h incubation at rt in the dark. To this was added 1 µL 200 mM sodium sulfite incubated for 20 min. at rt. After adding 12 µL 50 mM sodium acetate buffer (pH 4), 5 µL of 20 mM ethylenediamine hydrochloride (pH 7.2) was added to the RNA. The reaction mixture was incubated for 1 h at rt, then for 1 hour at 37 °C. Then 2 µL freshly prepared 200 mM sodium cyanoborohydride in ACN was added to the solution and incubated for 30 min. at rt and precipitation was effected with 2 volumes of 2% lithium perchlorate in acetone for 1 h at 0 °C. The sample was centrifuged at 12,100 g for 60 min. at 4°C. After removal of the supernatant

the RNA pellet was washed once with acetone and air dried. The light yellow pellet of RNA was taken up in 7.5 µL water. To this was added 0.75 µL 1 M borate buffer, pH 8.6. Dabcyl NHS ester (~ 100 µg, 0.3 µmol) dissolved in 2 µL DMSO was added and mixed well by pipetting up and down. The reaction was left for 6 hours in the dark at rt and then at 50 °C for 2 hours. Precipitation was done with NaOAc and EtOH. HPLC gradient 3% B for 4 min. to 40% B in 40 min.: t_R = 20 min. Yield: 30 pmol, 10%. MALDI-TOF calc. $[M-H]^-$ 20291.9, found 20294.4.

FAM pre-let-7 using FAM-ApG

5′-(5/6)FAM-AGGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUC AUACUAUACAAUGUGCUAGCUUUCUUUGCUC-DABCYL-3′ (32): An *in* vitro transcription was carried out according to protocol H with the addition of 2.2 mM 5′-(5/6)-(5/6)FAM-ApG transcription starter in a final volume of 45 µL. The following DNA template was used:

5′-AGCAAAGAAAGCTAGCACATTGTATAGTATGATGTGTAATTACTACTATACTTCCT ACTACCTCAATTTGCC**TATAGTGAGTCGTATTA**-3′. HPLC gradient 3% B for 4 min. to 20% B in 20 min.: t_R = 20/21 min., both isomers. Yield: 39%, 0.84 nmol (percent labeled full length transcript). MALDI-TOF calc. $[M-H]^-$ 23909.1, found 23015.7. To the FAM-labeled transcript in 350 µL (20.0 µg, 0.84 nmol) was added 140 µL DMSO (25%), 56 µL 10x T4 RNA ligase buffer, 4 µL 50mM DABCYL pCp **39**, and 12 µL T4 RNA ligase I (240 U) and mixed well. The reaction was left for 1 hour at 37 °C at which point an additional 10 µL ligase was added (200 U) and the reaction incubated overnight at 16 °C. The reaction was worked up as described in protocol G. The 3′-phosphate was removed with CIP as described. HPLC gradient 3% B for 4 min. to 40% B in 40 min.: t_R = 28 min. Yield: 90 pmol, 10%. MALDI-TOF calc. $[M-H]^-$ 24508.6, found 24505.8

FAM only pre-mir-21 using amino APG and FAM NHS-ester

5′-(5/6)FAM-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACA CCAGUCGAUGGGCUUGG-OH-3′ (33): An *in vitro* transcription was carried out according to protocol H with the addition of 1 mM 5′-amino-ApG transcription starter in a final volume of 20 µL. The following DNA template was used:

5′-$C_{OMe}C_{OMe}$AAGCCCATCGACTGGTGTTGCCATGAGATTCAACAGTCAACATCAGTC TGATAAGCC**TATAGTGAGTCGTATTA**-3′. The subscript -OMe signifies 2′-*O*-methylation. The crude product was then taken up in 220 µL 100 mM sodium borate buffer, pH 8.6. (5/6)FAM NHS ester (1 mg, 2 µmol) was dissolved in 35 µL DMSO. This was added to the RNA. The clear yellow solution was mixed well and allowed to react for 2 hours at 50 °C in the dark while being shaken. The reaction mixture was precipitated with NH_4OAc and EtOH. HPLC gradient 3% B for 4 min. to 20% B in 40 min.: t_R = 31/32 min., both isomers. Yield: 0.5 nmol, 22% (percent labeled full length). MALDI-TOF calc. $[M-H]^-$ 19819.1, found 19821.9.

FAM pre-mir-21 using FAM-ApG and pCp-DABCYL ligation

5′-(5/6)FAM-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACA CCAGUCGAUGGGCUUGGC-DABCYL-OH-3′ (34): An *in vitro* transcription was carried out according to protocol H with the addition of 2.2 mM 5′-(5/6)FAM-ApG transcription starter in a final volume of 45 µL. The aforementioned DNA template was used. After PCI workup EtOH precipitation (NH_4OAc) the crude product was directly labeled with DABCYL pCp **39**. The crude FAM-labeled transcript in 50 µL 1 mM ammonium citrate buffer was combined with 25 µL DMSO (25%), 10 µL 10x T4 RNA ligase buffer, 7.5 µL water, and 5 µL 50 mM DABCYL pCp **39**. After denaturing for 2 min. at 95 °C with snap cooling on ice 2.5 µL RNAse inhibitor (100 U) and 5 µL T4 RNA ligase I (100 U) were added and the reaction left 11 °C for 16 hours. After PCI workup and ethanol precipitation the crude product was purified by HPLC (3% B for 4 min. to 40% B in 40 min.). Fractions containing fully labeled product **34** (0.11 nmol, t_R = 27 min.) as well as FAM-labeled transcript (0.58 nmol, t_R = 20/21 min., both isomers) and unlabeled transcript (t_R = 19 min.) were collected. The remaining FAM-labeled transcript was ligated with DABCYL pCp **39** as described. After PCI workup and ethanol precipitation (NaOAc) a combined total of 0.68 nmol DABCYL-labeled product was obtained without further HPLC purification needed as determined by MALDI-TOF analysis. The 3′-phosphate was removed as follows: In a total volume of 50 µL was combined RNA, 50 mM HEPES, pH 6.5, 5 mM $MgCl_2$, 5 mM DTT, 20 U RNAse inhibitor, and 50 U PNK. The reaction was incubated at 37 °C for 4 hours. PCI workup and ethanol precipitation gave **34** in 74% yield (0.50 nmol). MALDI-TOF calc. $[M-H]^-$ 20420.0, found 20431.6.

TMR pre-mir-21 using TMR-ApG and pCp-DABCYL ligation

5'-(5/6)TMR-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUUGGC-DABCYL-3' (35): An *in vitro* transcription was carried out according to protocol H with the addition of 2.5 mM 5′-(5/6)TMR-ApG transcription starter in a final volume of 40 µL. The aforementioned DNA template was used. The crude product was HPLC purified as described (3% B for 4 min. to 40% B in 40 min., t_R = 26/29 min., both isomers, 0.2 nmol, 9% labeled full length transcript). The TMR-labeled transcript (98µL in water) was combined with 15 µL 10x T4 RNA ligase buffer, 30 µL DMSO and then denatured (95 °C for 2 min. then on ice). RNAse inhibitor was added (2 µL, 80 U) followed by 2 µL DABCYL pCp **39**. The reaction vial was cooled on ice for 10 min. and then 3 µL T4 RNA ligase I (60 U) were added and the reaction incubated 11 °C for 20 hours. After PCI workup and ethanol precipitation (NaOAc) the product was taken up in a total volume of 50 µL containing RNA, 50 mM HEPES, pH 6.5, 5 mM $MgCl_2$, 5 mM DTT, 20 U RNAse inhibitor, and 50 U PNK. The reaction was incubated at 37 °C for 4 hours. PCI workup and ethanol (NH_4OAc) precipitation gave **35** in quantitative yield (0.20 nmol). MALDI-TOF calc. $[M-H]^-$ 20474.8, found 20467.5.

5.3.4 Cell Biology

5.3.4.1 Cell Culture

HEK 293 cells were handled according to a slow freeze / fast thaw approach, whereby cells for long-term storage in liquid nitrogen are slowly frozen at -20 °C before placing in liquid nitrogen. Frozen stocks are then quickly thawed at 37 °C and added directly to medium containing serum to stimulate growth. Cells were stored in 8% DMSO with 42% medium and 50% FCS. D-MEM containing 1% penicillin-streptamycin antibiotic mix and 10% FCS was used for growth of cells ('growth medium'). Cells were regularly passaged (1:10) and kept under sterile conditions at 5% CO_2 and 95% humidity.

Storage of cells: Briefly, confluent cells from a 75 cm^2 culture flask were rinsed with PBS and treated for 3 min. with 1 mL trypsin-EDTA solution at 37 °C. After quenching with 9 mL medium the cells were centrifuged and the pellet taken up in 2 mL FCS. To this was added slowly 2 mL of 16 % DMSO in medium (0.9 mL DMSO / 4.1 mL serum-free medium) and mixed well. 1 mL aliquots were frozen first at -20 °C and then placed in liquid nitrogen.

Thawing of cells: A 1 mL aliquot of cells was warmed in the hand until liquid / slush. This was quickly added to a 75 cm^2 culture flask containing 20 mL growth medium and mixed by swirling till completely dissolved and left overnight in the CO_2 incubator before changing the medium.

Harvesting of cells for cell lysate: Cells at 90-100% confluency were rinsed with PBS and treated with 1 mL trypsin-EDTA (or 0.25% trypsin) for 3 min. at 37 °C. Then 9 mL growth medium were added and mixed well. From this 1 mL (or less) was added to a fresh 75 cm^2 culture flask containing 25 mL growth medium and replaced in the CO_2 incubator. The remaining volume was transferred to a 15 mL tube and centrifuged at 2,000 g for 10 min. at 4 °C. The supernatant was removed and the cells resuspended in several mL PBS and again centrifuged as before. After removing the supernatant the cells were resuspended in 1 mL storage buffer (20 mM Tris-HCl, pH 7.6, 75 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 10% Glycerol und Roche protease inhibitor). The cells were disrupted with ultrasound at 4 °C for 5 min. and 40% constant power. The disrupted cells were centrifuged as before. The supernatant was transferred to a 1 mL PP reaction vial and centrifuged once more at 10,000 g for 10 min. at 4 °C. The supernatant was transferred to a fresh 1.5 mL PP reaction vial and the protein concentration determined *via* a Bradford assay (100-250 µg/mL).[216] The cell lysate was stored at -20 °C until further needed. The cell lysate for control measurements was heated at 95°C for 20 min. and also stored at -20°C.

5.3.4.2 Transfection of Cells

This protocol describes in detail the knockdown of Dicer in HEK 293 cells using siRNA specific for Dicer mRNA before observing beacon cleavage *via* fluorescence microscopy. AS a control siRNA without complementarity to Dicer mRNA was used (control siRNA). For experiments where no knockdown was performed please go directly to **Step 4** below. In all experiments medium used for the transfection reagent / RNA mix contained no serum, no antibiotics, and no phenol red (‘plane medium’, Biochrom). During transfection cells were placed in D-MEM without phenol red containing only 2.5% FCS and no antibiotics (‘transfection medium’). After 4 hours transfection time medium was replaced with transfection medium until the next transfection day. All RNA (control siRNA, Dicer siRNA,

and all beacons) was transfected at a final concentration of 9 nM. All RNA samples for transfection were diluted to a final concentration of 2 µM in Dicer reaction buffer and renatured by heating for 5 min. at 95 °C and then cooling over 24 min. to rt.

Step 1: On day -1 cells at 90-100 % confluency were trypsinized as described previously and passaged into 9.6 cm^2 petri-dishes with glass bottoms containing 2 mL growth medium (MatTek) at 1:10 ratio (calculated for smaller surface area). Cells were left overnight before transfection.

Step 2: On day +1 medium was changed to 1 mL transfection medium. Cells were transfected using Roche X-tremeGene siRNA transfection reagent (#04 476 093 001) according to the manufacturer's instructions. Briefly, 2.5 µL transfection reagent was added to 47.5 µL plane medium for a total volume of 50 µL. To this was added 5 µL 2 µM RNA (or buffer) in 45 µL plane medium and incubated for 20 min. The entire 100 µL volume was then added to a single petri-dish containing cells in 1 mL transfection medium to give a final concentration of RNA of 9 nM and swirled gently. After 4 hours transfection time the medium was replaced with 2 mL transfection medium and left in the CO_2 incubator until the next transfection.

Step 3: On day +3 the cells were once again transfected as described in step 2.

Step 4: On day +4 the cells were transfected as described in step 2, except that now the beacon or other RNA was transfected instead of the Dicer siRNA. After transfection the fluorescence was observed over the course of 1-24 hours (see section 5.3.5). The medium was not changed during fluorescence measurements.

5.3.4.3 Western Blot

A Western Blot of knockdown HEK 293 cells and untreated cells along with a control (empty transfection, i.e. only buffer) was performed. Facilities in the working group of Prof. Andreas Herrmann from the Humboldt-Universität zu Berlin were used. Two samples each of knockdown cells, untreated cells and one empty transfection were harvested from a 6-well plate similarly as described under "Harvesting of cells for cell lysate" (5.3.4.1 Cell Culture) except that the cells were disrupted in Dicer reaction buffer and a smaller buffer volume was used. The disrupted samples were centrifuged and the total protein concentration of the supernatants determined by a Bradford assay and then immediately frozen at -20 °C. The amount of sample was adjusted to obtain approximately equal total protein concentrations amongst samples (6 µg per sample total protein). The largest possible volume (20 µL) was

used as to maximize signal. To each sample was added 1 µL 50 mM phenylmethylsulfonyl fluoride to inhibit proteases. Samples were denatured with 10 µL SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) at 98 °C for 10 min. and then loading onto a 10% SDS gel and run at 150 V for 50 min. (running buffer: Tris-glycine, pH 8.3). The proteins were transferred onto Bio-Rad blotting paper using a Bio-Rad semi-dry transfer apparatus (transfer buffer: 0.5x SDS running buffer, 25% MeOH, 0.05% SDS) at 15 V for 30 min.

Blocking: The membrane was blocked with blocking buffer (1x PBS, 5% powdered skim milk, 0.5% Tween 20) for 1 hour.

Primary Antibody: Primary rabbit anti-Dicer antibody (Ambion) was diluted 1:5,000 in blocking buffer and incubated for 1 hour with the membrane and then washed 3 x 10 min. with blocking buffer.

Secondary Antibody: Secondary goat anti-rabbit peroxidase antibody (Sigma-Aldrich) was diluted 1:10,000 in blocking buffer and incubated with the membrane for 1 hour and then rinsed 3 x 10 min. with blocking buffer and once with PBS.

Chemiluminescence reaction: Enhanced chemiluminescence ECL detection solution (Amersham / GE Healthcare) was mixed according to the manufacturer and incubated for 5 min. with the membrane.

Detection: The membrane was detected using photographic film (Amersham/GE) for 2, 5 and 10 min.

5.3.5 Fluorescence Microscopy

All fluorescence microscopy work was done in the working group of Prof. Andreas Herrmann.

5.3.5.1 Epifluorescence Measurements

Images were taken with an Olympus IX-81 inverted microscope (Tokyo, Japan) equipped with an 60 x (N.A 1.35) oil-immersion objective at rt. Wavelengths (ex/em) of 470-490 / 510-550 nm for FAM (U-MNIBA2 filter) and 530-550 / >590 nm (U-MNG2 filter) for Cy3 were applied. Measurements were made at the given time points and samples kept at 37 °C in-between measurements. Images were evaluated using MetaView software (Universal Imaging,

Buckinghamshire, England). Documented images were processed using ImageJ software (v. 1.38, Wayne Rasband, NIH, USA).

5.3.5.2 Confocal Fluorescence Measurements

Images of the equatorial plane of the cells were taken by confocal laser scanning microscopy. Images were taken with an inverted IX-81 fluorescence microscope equipped with an Olympus Fluoview 1000 scanhead (Tokyo, Japan) and a 60 x (N.A. 1.35) oil-immersion objective. An ACU Climatization Control climate chamber (Evotec Technologies, Hamburg, Germany) was used at 95 % humidity and 37 °C where stated. FAM fluorescence was excited with a 488 nm Ar-ion laser, while Cy3 fluorescence was excited with a 543 nm He–Ne laser. FAM emission was recorded between 500-530 nm. Cy3 emission was recorded >590 nm. Measurements were made at the given time points and samples kept at 37 °C in-between measurements or samples were climate controlled. Images were evaluated using MetaView software (Universal Imaging, Buckinghamshire, England). Documented images were processed using ImageJ software (v. 1.38, Wayne Rasband, NIH, USA).

5.3.6 List of Buffers and Solutions

3 M NaOAc, pH 5.2

5 M NH_4OAc, pH 7.5

1 mM Ammonium citrate, pH 6.4 or 7.0

TE buffer: 10 mM Tris-HCl (pH 7.4 or variable), 1 mM EDTA (pH 8.0)

CIP reaction buffer: 50 mM Tris-HCl, 100 mM NaCl 10 mM $MgCl_2$, 1 mM DTT, pH 7.9

PNK reaction buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP, pH 7.8

Ligation reaction buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.8

Dicer reaction buffer: 20 mM Tris-HCl, pH 6.8, 12.5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT

Dicer enzyme dilution buffer (can also be used for cell lysate with the addition of protease inhibitor): 100 mM NaCl, 20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM DTT, 0.1% Triton X-100, and 10% glycerol

Cell lysate buffer: 20 mM Tris-HCl, pH 7.6, 75 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 10% Glycerol und Roche protease inhibitor without EDTA

TBE buffer: Tris / borate / EDTA 0.89 M Tris-borate and 20 mM EDTA

6x Sucrose loading buffer: 18 mM EDTA, 0.05% bromophenol blue, 40% sucrose

1-2X Formamide loading buffer: 90% formamide, 18 mM EDTA, and 0.05% bromophenol blue

SDS loading buffer: 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol

SDS running buffer: 25 mM Tris,250 mM glycine, pH 8.3, 0.1% SDS

SDS transfer buffer: 0.5x SDS running buffer, 25% MeOH

PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

Blocking buffer: 1x PBS, 5% powdered skim milk, 0.5% Tween 20

Growth medium: D-MEM containing 10% FCS and 1% penicillin-streptomycin

Plane medium: D-MEM without phenol red and no FCS or antibiotics

Transfection medium: D-MEM without phenol red containing 2.5% FCS, but without antibiotics

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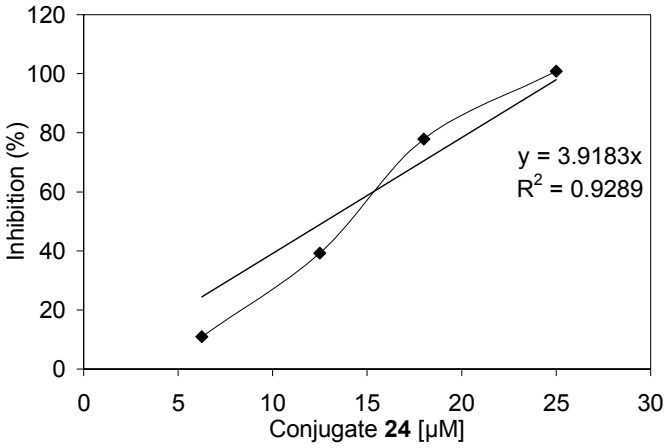
Supplements

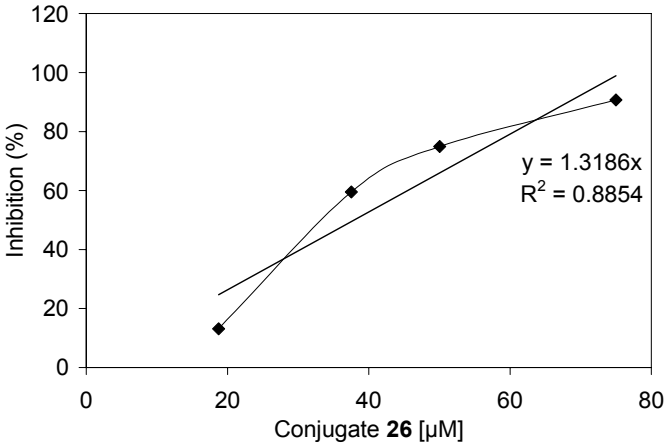
IC_{50} Calculations

All IC_{50} values were calculated between 20-120 min. from triplicate measurements.

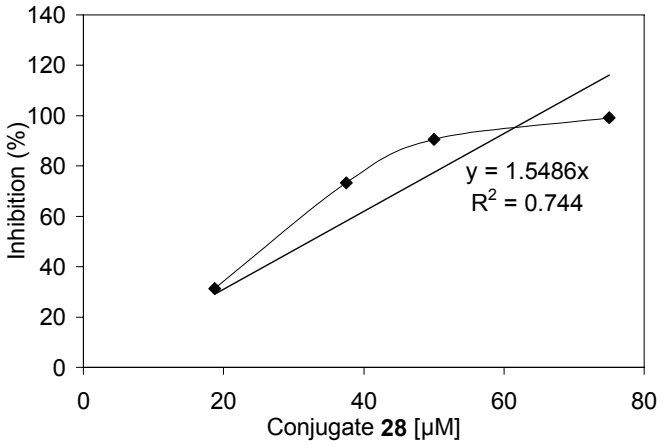
Conjugate **24** with pre-let-7 beacon **10**



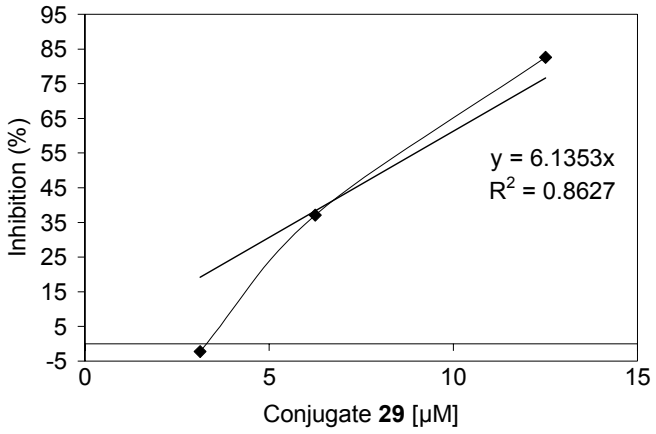
Conjugate **26** with pre-let-7 beacon **10**



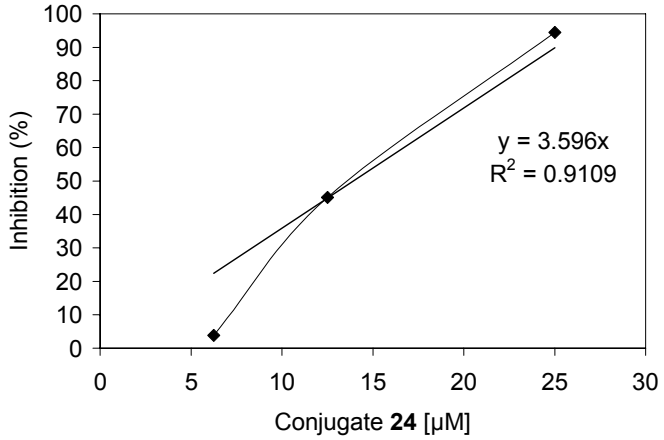
Conjugate **28** with pre-let-7 beacon **10**



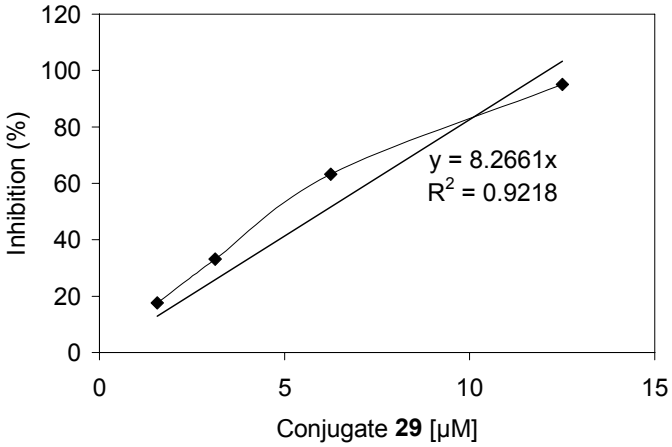
Conjugate **29** with pre-let-7 beacon **10**



Conjugate **24** with pre-bantam beacon **17**

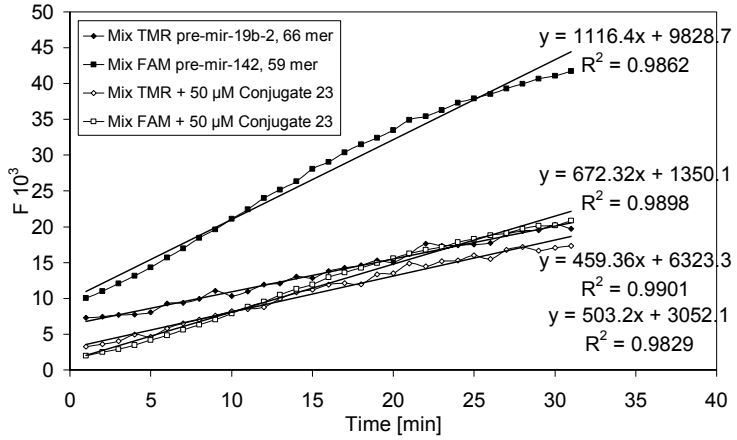


Conjugate **29** with pre-bantam beacon **17**

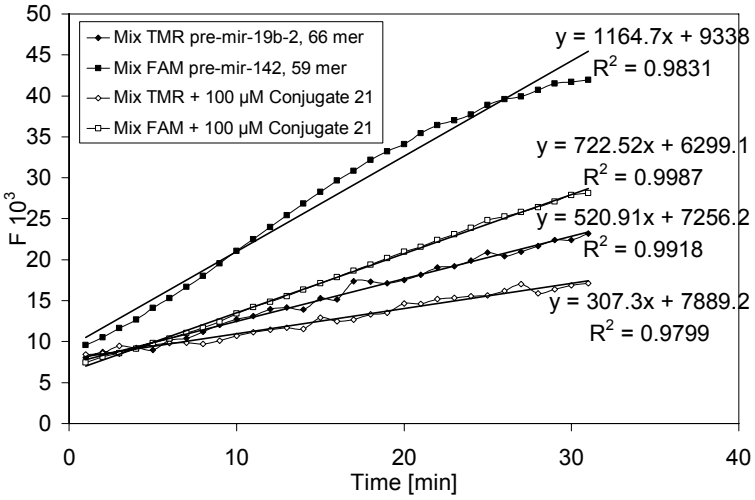


Inhibition Values (Table 4)

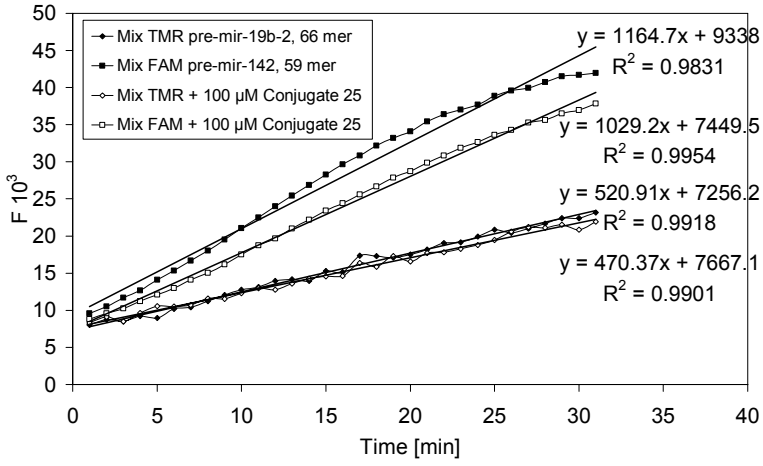
Conjugate **23**



Conjugate **21**

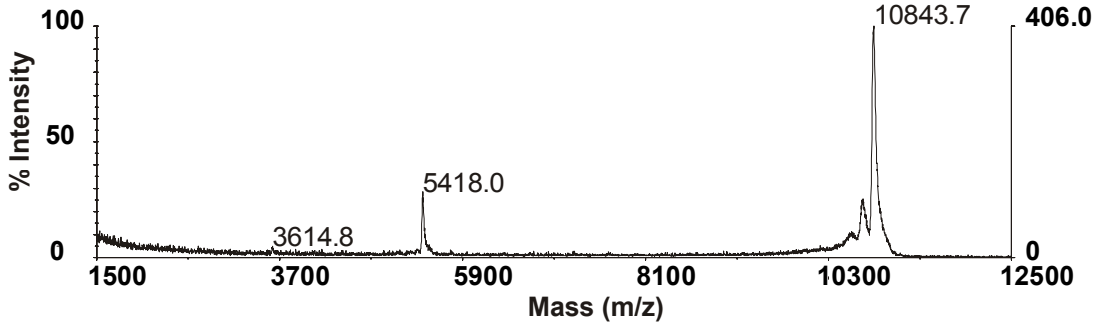


Conjugate **25**

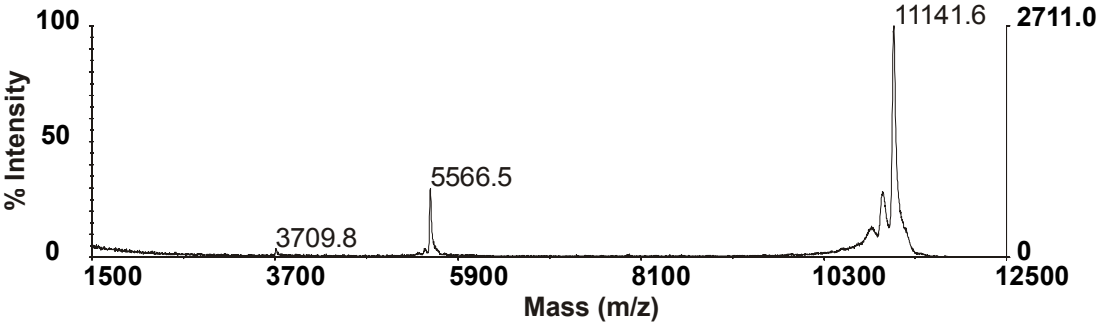


MALDI-TOF Spectra of Synthetic RNA

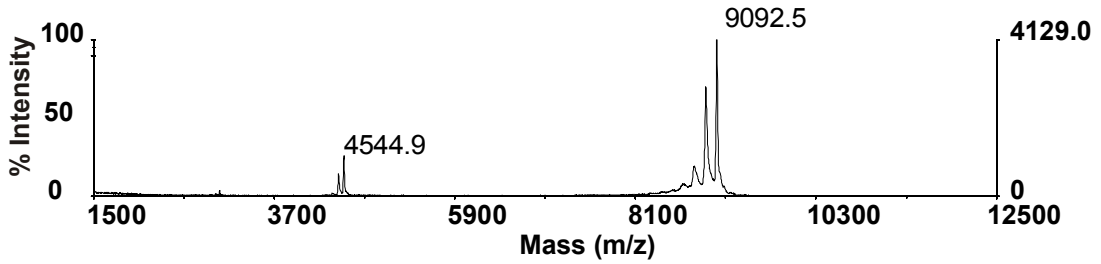
Pre-mir-142 5′-(6)FAM-CAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′, 32 mer (1)



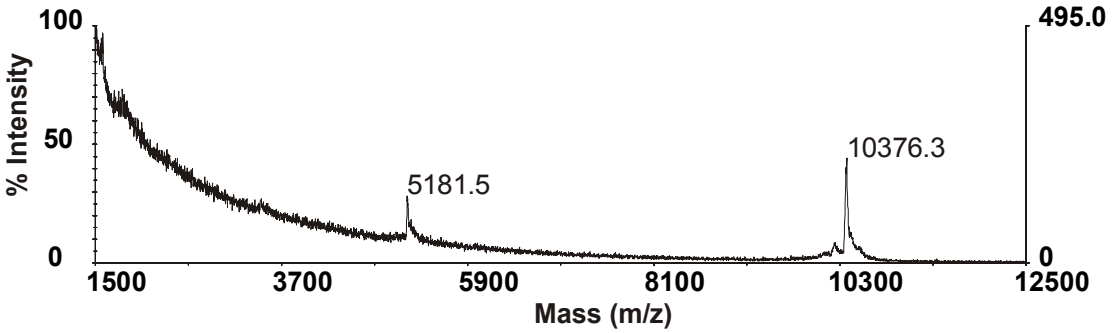
Pre-mir-142 5′-(6)FAM-CCAUAAAGUAGAAAGCACUACUAACAGCACUGG-OH-3′, 33mer (2)



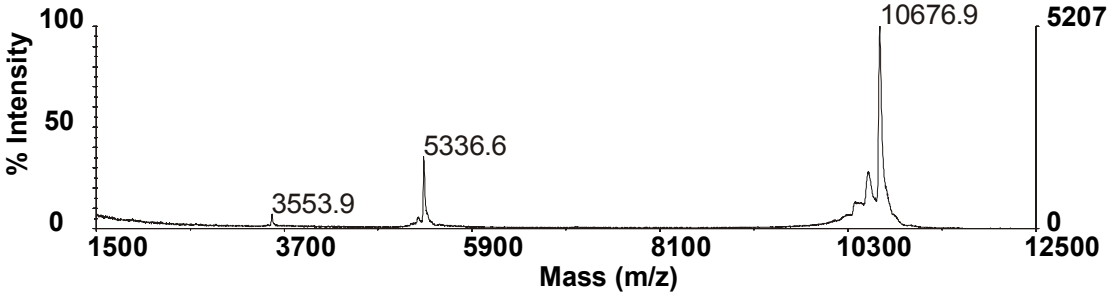
Pre-mir-142 5′-PO_3-AGGGUGUAGUGUUUCCUACUUUAUGGdU-DABCYL-3′, 27 mer (3)



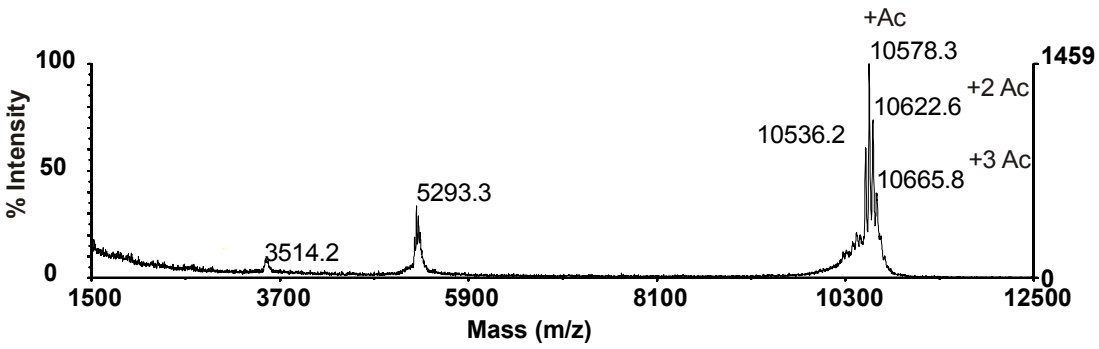
Pre-mir-19b-2 5′-Cy3-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 31 mer (4)



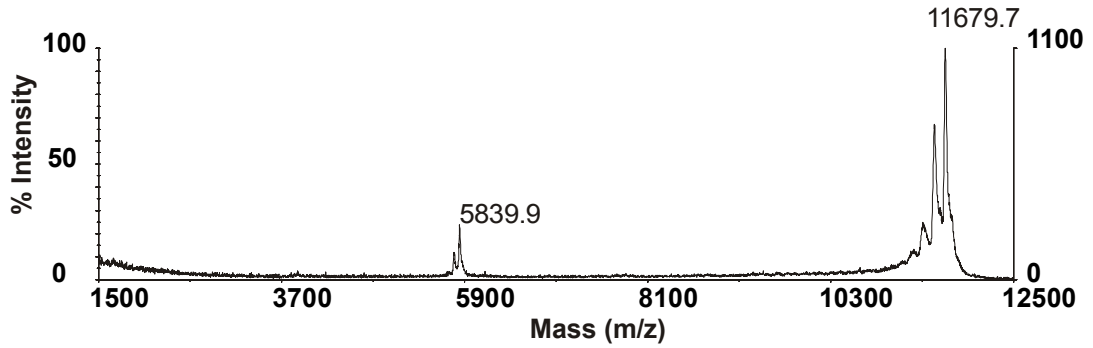
Pre-mir-19b-2 5′-Cy3-CAGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 32 mer (5)



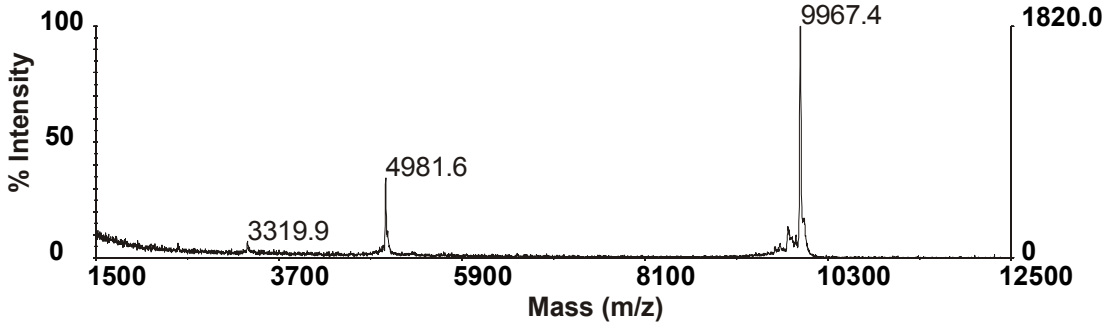
Pre-mir-19b-2 5′-(6)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUA-OH-3′, 31 mer (6)



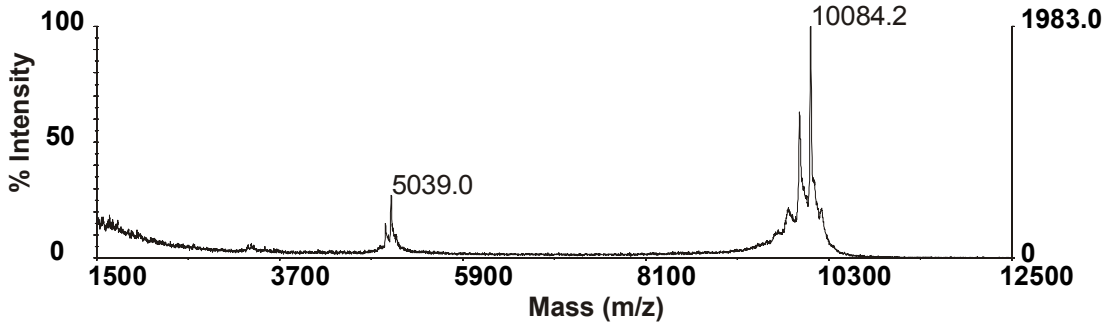
Pre-mir-19b-2 5′-PO_3-UGUAUAUGUGGCUGUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′, 35 mer (7)



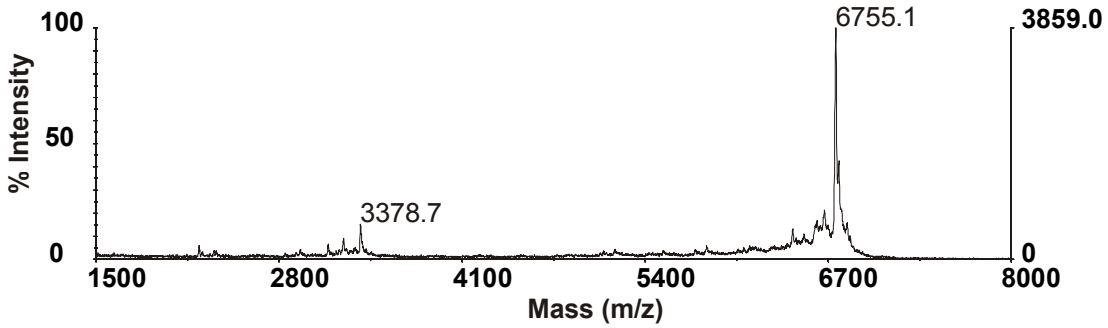
Pre-bantam 5′-(6)FAM-CCGGUUUUCGAUUUGGUUUGACUGUUUUUC-OH-3′, 30 mer (8)



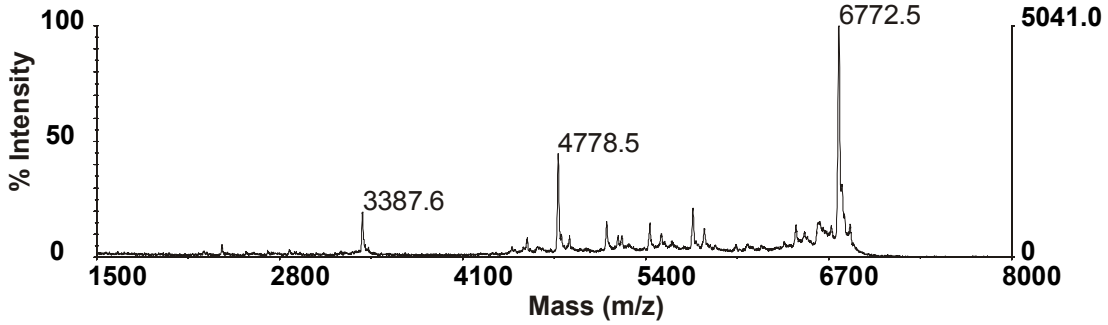
Pre-bantam 5′-PO_3-AUACAAGUGAGAUCAUUUUGAAAGCUGAUdU-DABCYL-3′, 30 mer (9)



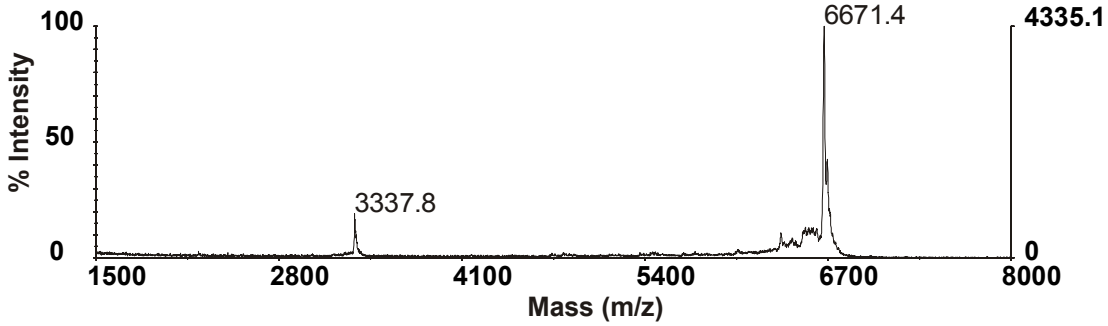
Dicer siRNA-1 5′-PO_3-UCCAGAGCUGCUUCAAGCAGU-OH-3′ (55)



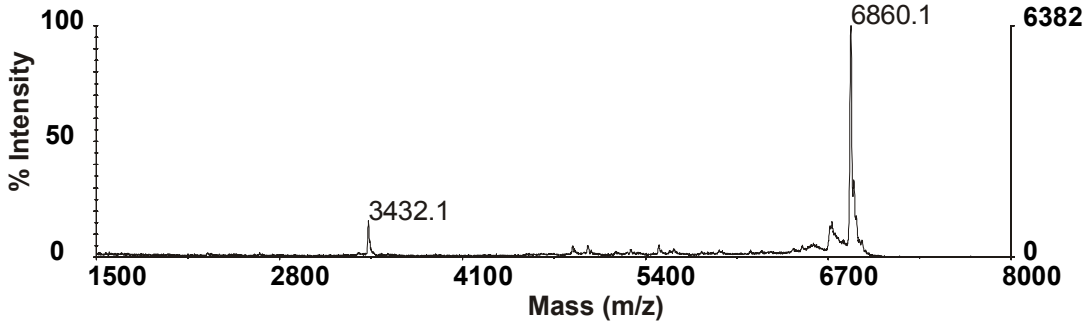
Dicer siRNA-2 5′-PO_3-UGCUUGAAGCAGCUCUGGAUC-OH-3′ (56)



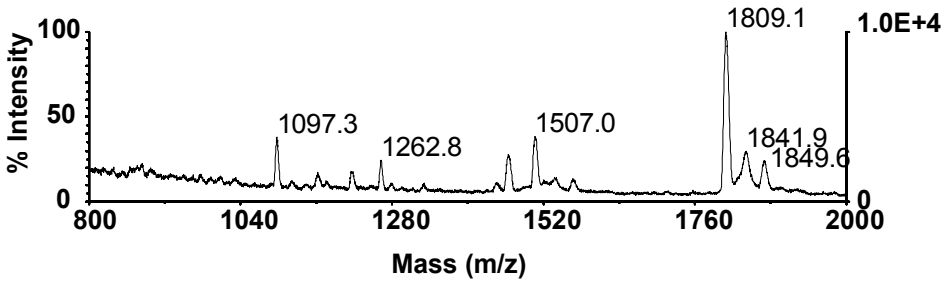
Control siRNA-1 5′-PO_3-CUUUAAGCUCCCUGAGCGUUU-OH-3′ (57)



Control siRNA-2 5′-PO_3-ACGCUCAGGGAGCUUAAAGUG-OH-3′ (58)



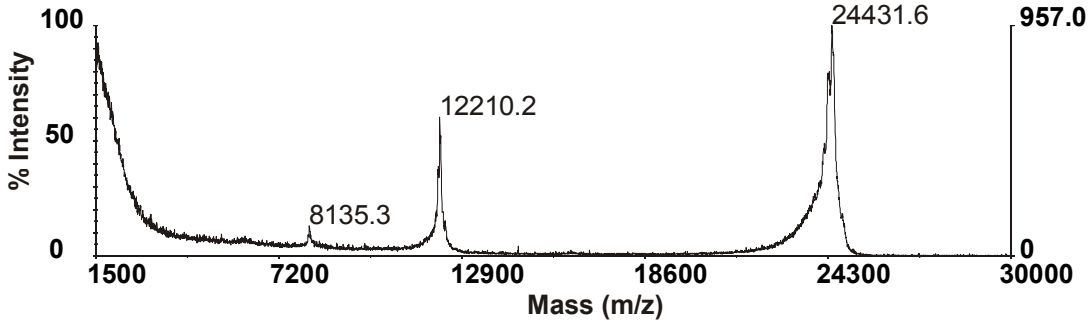
5 mer 5′-DMT-$U_HU_{SH}U_HU_{SH}$G-OH-3′ (46)



MALDI-TOF Spectra of Synthetic Beacons

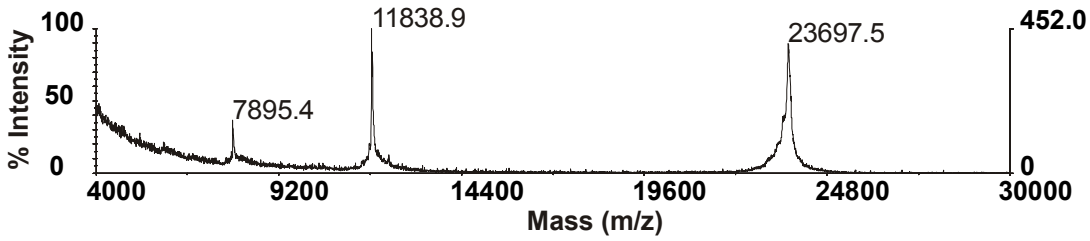
FAM pre-let-7, 73 mer

5′-(6)FAM-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAU ACUAUACAAUGUGCUAGCUUUCUUUGCUdC-DABCYL-3′ (10)



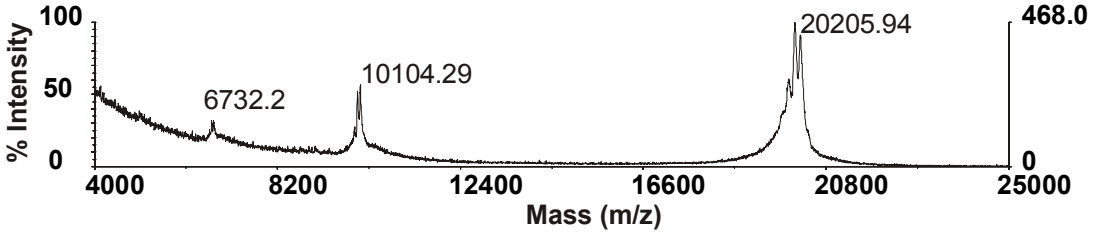
FAM-only pre-let-7, 72 mer

5′-(6)FAM-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAU ACUAUACAAUGUGCUAGCUUUCUUUGCU-OH-3′ (11)



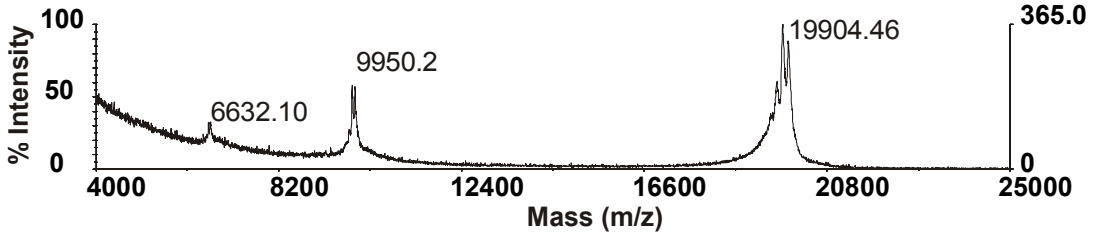
FAM pre-mir-142, 60 mer

5′-(6)FAM-CCAUAAAGUAGAAAGCACUACUAACAGCACUGGAGGGUGUAGUG UUUCCUACUUUAUGGdU-DABCYL-3′ (12)



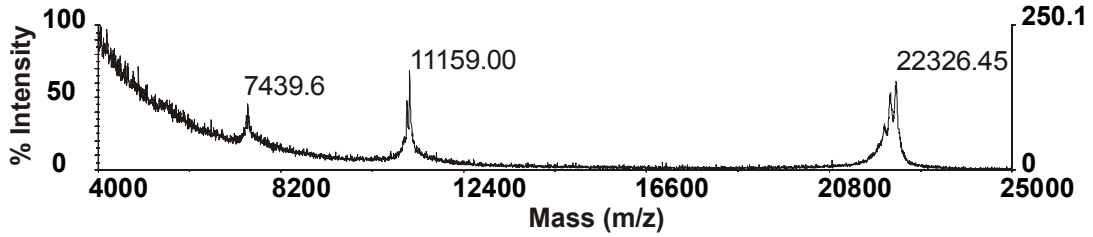
FAM pre-mir-142, 59 mer

5′-(6)FAM-CAUAAAGUAGAAAGCACUACUAACAGCACUGGAGGGUGUAGUGU UUCCUACUUUAUGGdU-DABCYL-3′ (13)



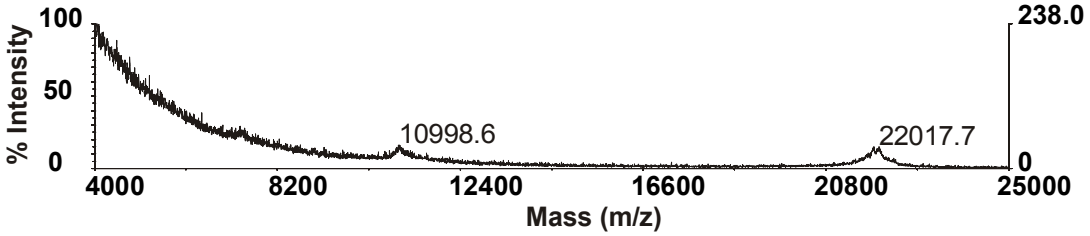
Cy3 pre-mir-19b-2, 67 mer

5′-Cy3-CAGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGU GCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (14)



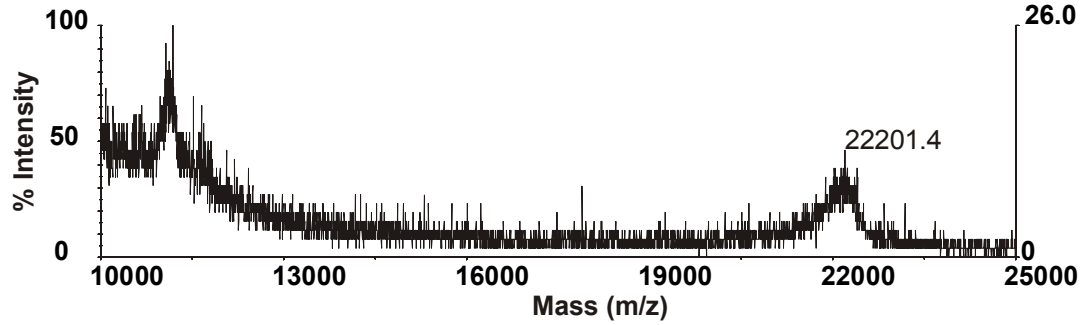
Cy3 pre-mir-19b-2, 66mer

5′-Cy3-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCUGUG CAAAUCCAUGCAAAACUGdU-DABCYL-3′ (15)



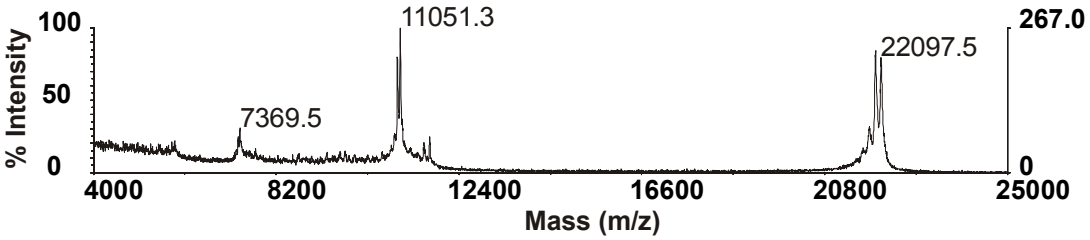
TMR pre-mir-19b-2, 66 mer self-made

5′-(6)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCU GUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (16)



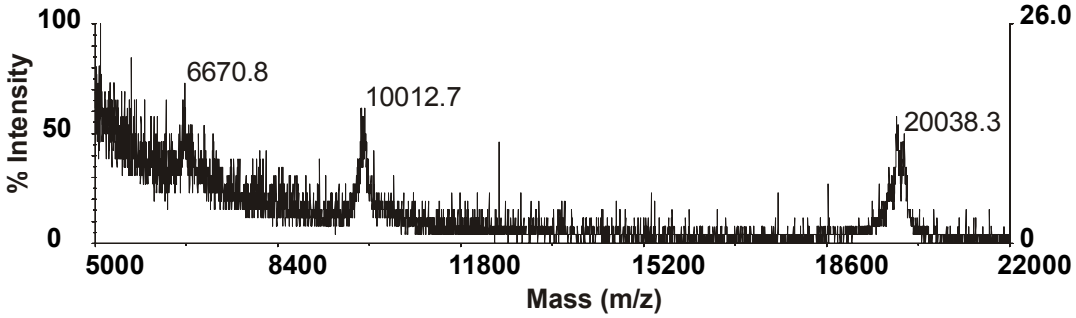
TMR pre-mir-19b-2, 66 mer made with IBA TMR 5′-strand

5′-(5)TMR-AGUUUUGCAGGUUUGCAUUUCAGCGUAUAUAUGUAUAUGUGGCU GUGCAAAUCCAUGCAAAACUGdU-DABCYL-3′ (16)



FAM pre-bantam

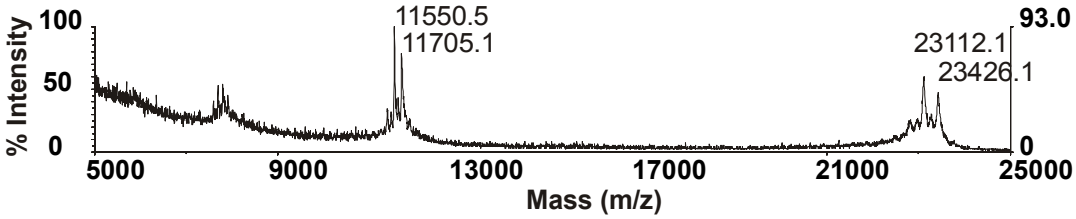
5′-(6)FAM-CCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGAUC AUUUUGAAAGCUGAUdU-DABCYL-3′ (17)



MALDI-TOF Spectra of *in vitro* Transcription RNA / Beacons

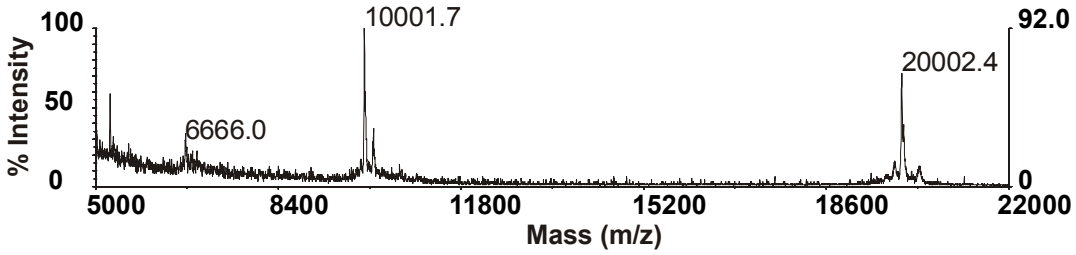
Let-7 pre-miRNA unlabeled

5′-PO_3-GGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUCAUACU AUACAAUGUGCUAGCUUUCUUUGCU-OH-3′ (62)



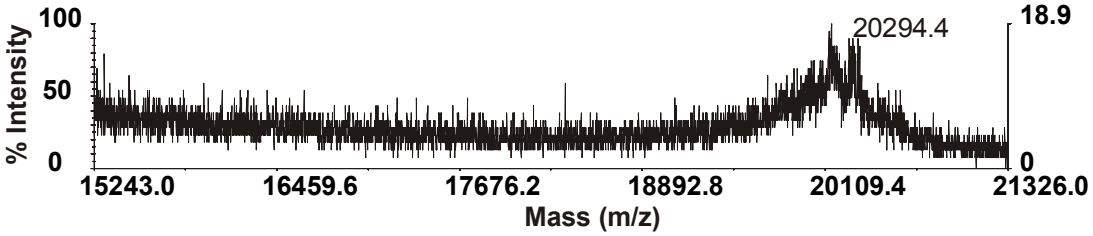
FAM-only pre-bantam using amino-ApG with FAM NHS-ester

5′-(5/6)FAM-AGCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGA UCAUUUUGAAAGCUGAUU-OH-3′ (30)



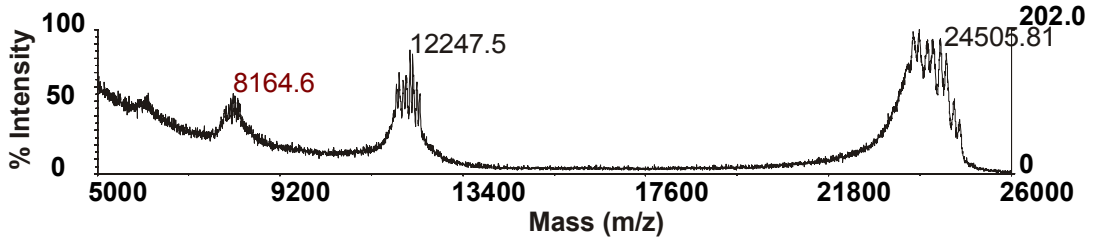
FAM-morpholino-DABCYL pre-bantam

5′-(5/6)FAM-AGCGGUUUUCGAUUUGGUUUGACUGUUUUUCAUACAAGUGAGA UCAUUUUGAAAGCUGAUU-DABCYL-3′ (31)



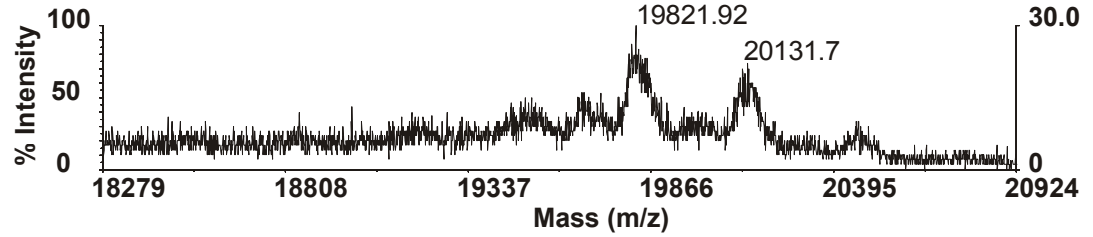
FAM pre-let-7 using FAM-ApG

5′-(5/6)FAM-AGGCAAAUUGAGGUAGUAGGUUGUAUAGUAGUAAUUACACAUC AUACUAUACAAUGUGCUAGCUUUCUUUGCUC-DABCYL-3′ (32)



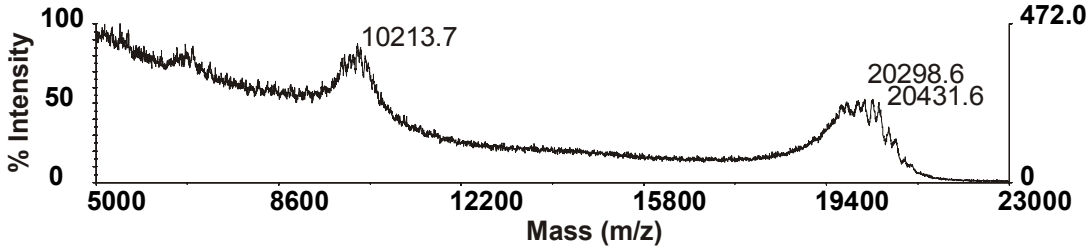
FAM-only mir-21 using amino-APG with FAM NHS-ester

5′-(5/6)FAM-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACA CCAGUCGAUGGGCUUGG-OH-3′ (33)



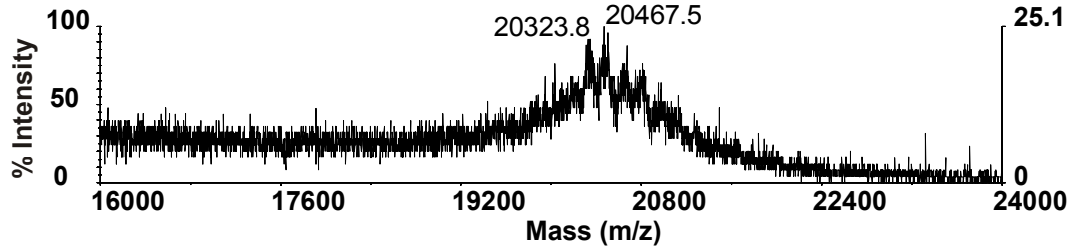
FAM mir-21 using FAM-ApG with pCp-DABCYL ligation

5′-(5/6)FAM-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACA CCAGUCGAUGGGCUUGGC-DABCYL-OH-3′ (34)



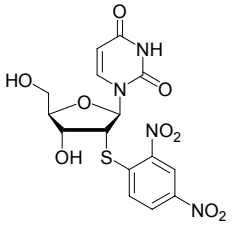
TMR mir-21 using TMR-ApG and pCp-DABCYL ligation

5′-(5/6)TMR-AGGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACA CCAGUCGAUGGGCUUGGC-DABCYL-OH-3′ (35)

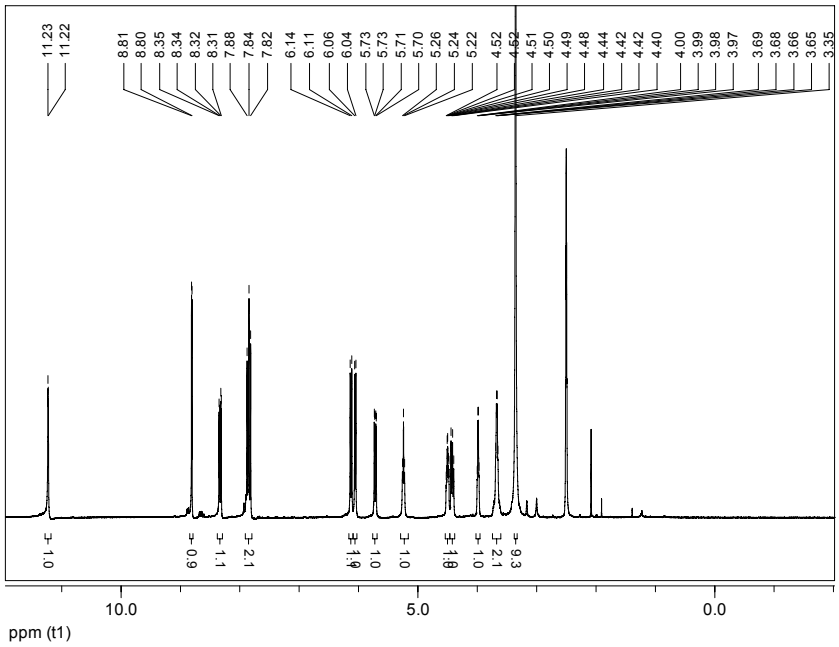


NMR Spectra

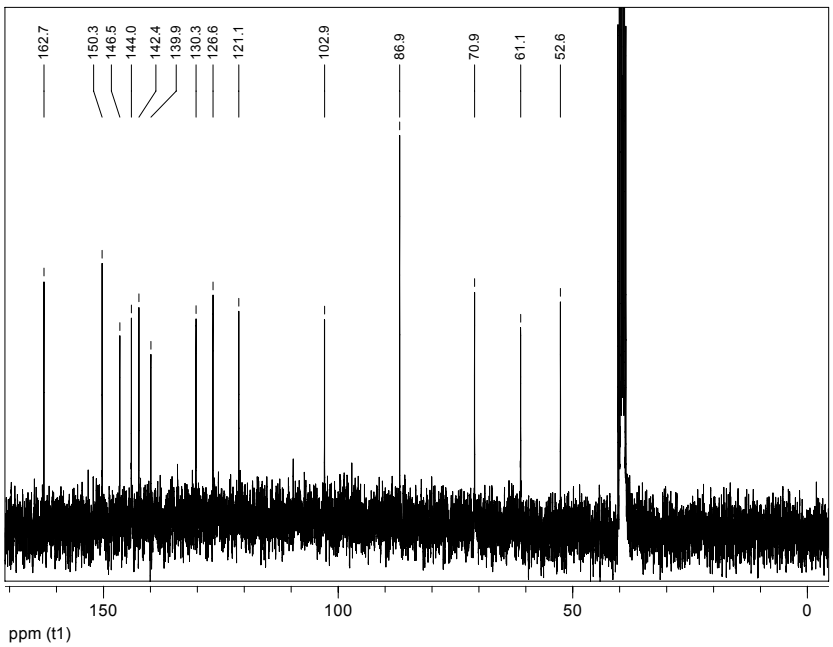
2′-Deoxy-2′-(2,4-dinitrophenylthio)uridine (43)



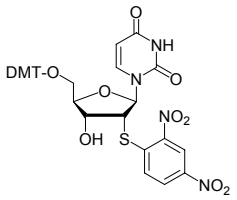
^{1}H (DMSO-d$_6$)



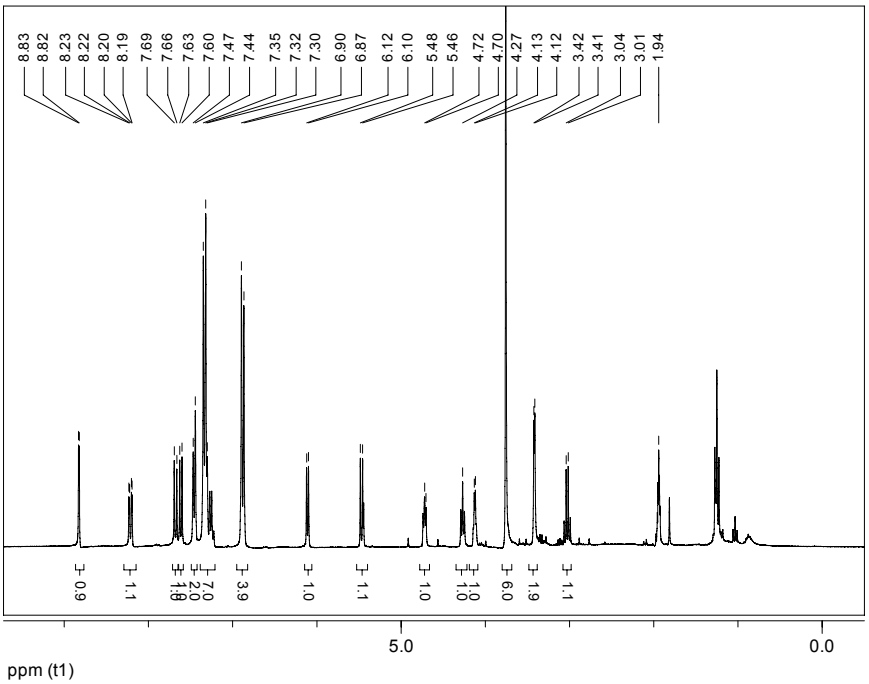
^{13}C (DMSO-d$_6$)



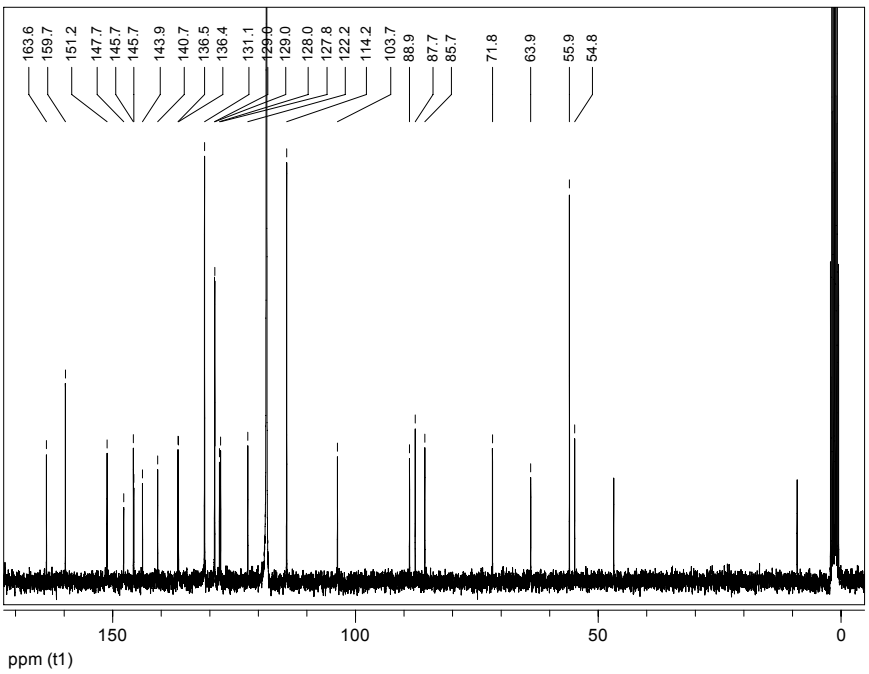
2′-Deoxy-2′-(2,4-dinitrophenylthio)-5′-*O*-(4,4′-dimethoxytrityl)uridine (44)



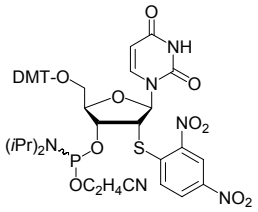
^{1}H (DMSO-d$_6$)



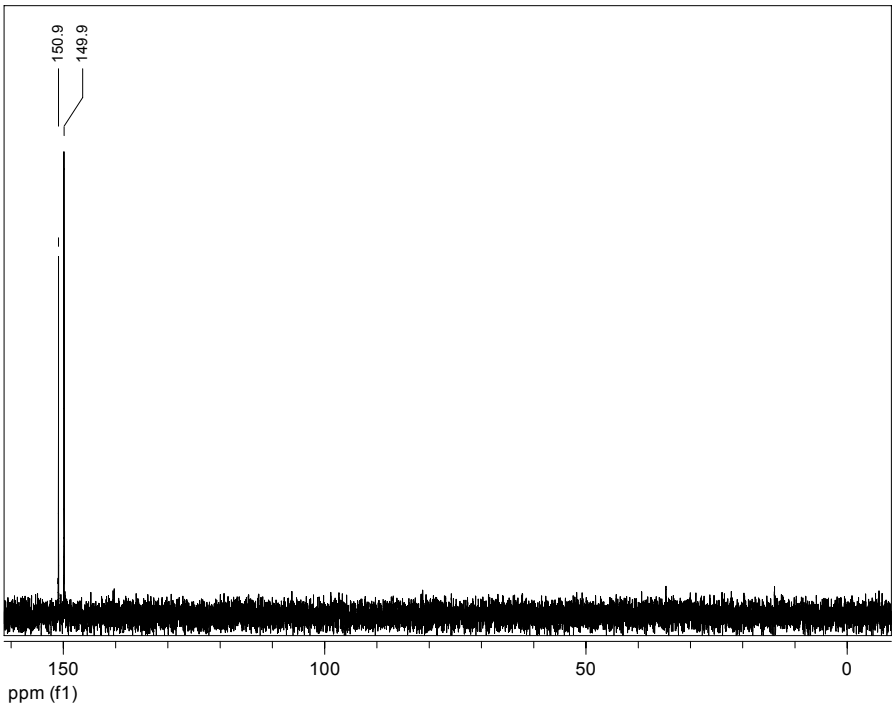
^{13}C (DMSO-d$_6$)



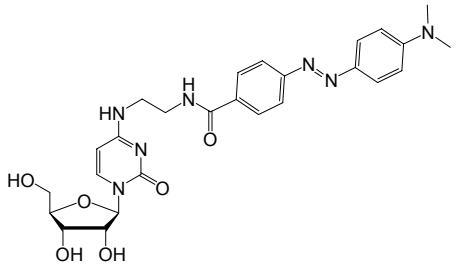
2′-Deoxy-2′-(2,4-dinitrophenylthio)-5′-*O*-(4,4′-dimethoxytrityl)uridine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylaminophosphoramidite) (45)



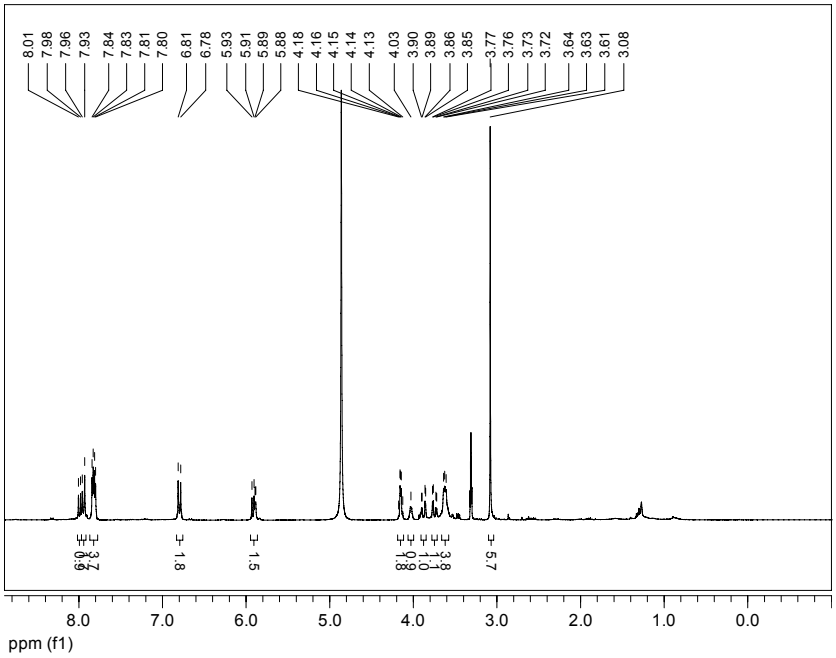
^{31}P (CD_3CN)



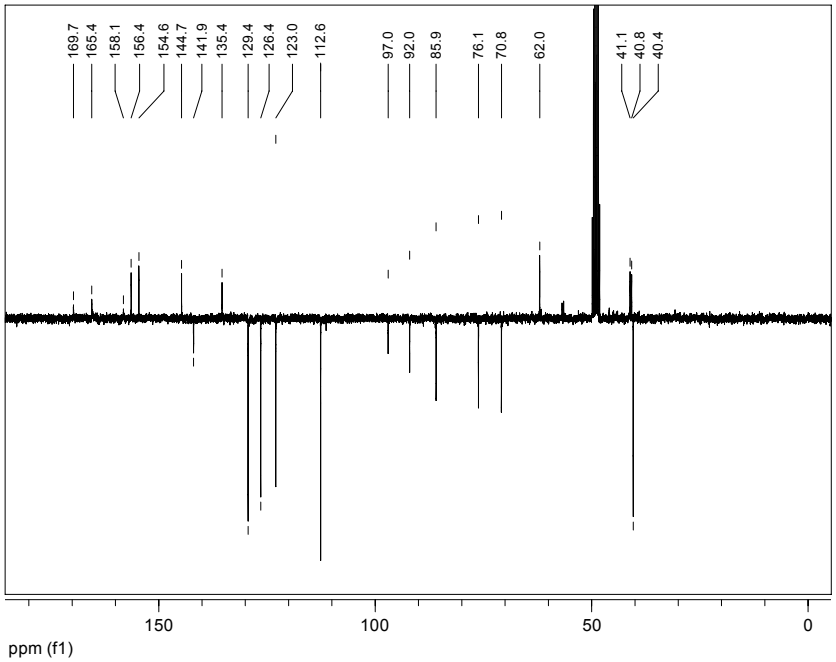
N^4-(4-(4-(dimethylaminophenylazo)benzamido)ethylcytidine (38)



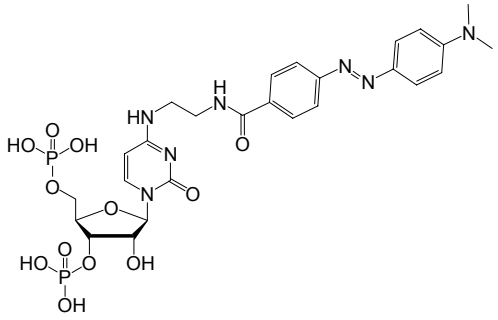
^{1}H (CD_3OD)



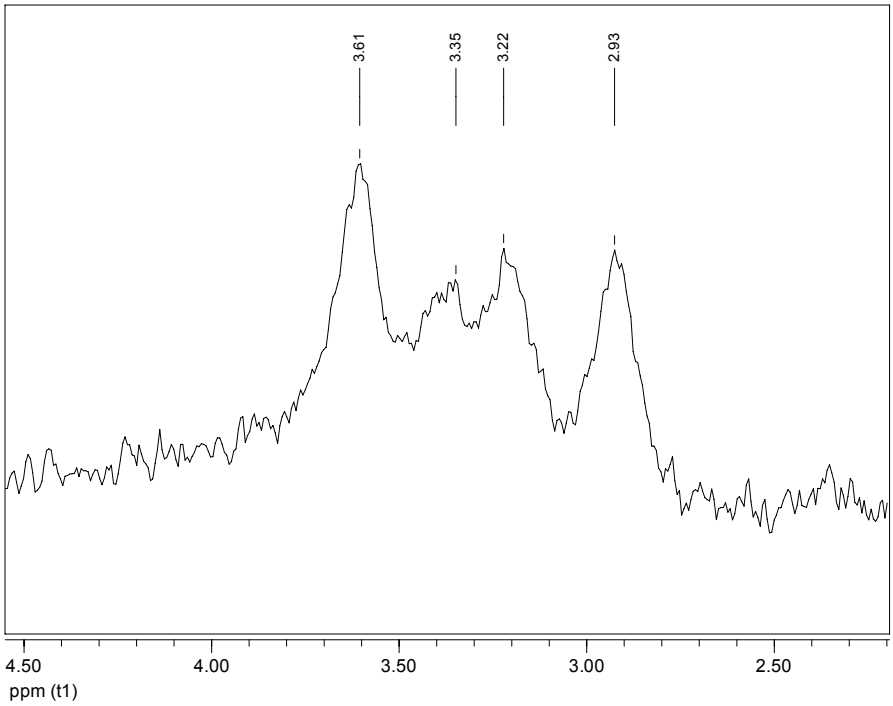
APT (CD_3OD)



N^4-(4-(4-(dimethylaminophenylazo)benzamido)ethylcytidine-3′(2′), 5′-bisphosphate (39)



^{31}P (D_2O)



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Last, but not least I have to thank my parents for their love and support over all these years.

Eidesstattliche Erklärung

Hiermit versichere ich, Brian Patrick Davies, die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel erarbeitet und verfasst zu haben.

Unterschrift: .....

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Berlin, im April 2008